Reactivity to Human Islets and Fetal Pig Proislets by Peripheral Blood Mononuclear Cells From Subjects With Preclinical and Clinical Insulin-Dependent Diabetes

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A simple, direct assay for T-lymphocyte reactivity to islet antigen(s) in human insulin-dependent diabetes mellitus (IDDM) should facilitate preclinical diagnosis and the evaluation of intervention therapy to avert autoimmune-mediated β-cell destruction. In subjects with preclinical or clinical IDDM, we measured the reactivity of peripheral blood mononuclear cells (PBMCs) incubated over 6 days with either adult human islets or fetal pig proislets, or other fetal pig tissues, and with human insulin. With islets, the stimulation index (SI) of [^{3}H]thymidine uptake by PBMCs exceeded the mean + 2SD of control subjects in 6 of 6 preclinical subjects (SI 8.7 ± 3.7), 7 of 11 clinical subjects (SI 5.2 ± 3.4), and 1 of 12 control subjects (SI 2.7 ± 1.7); with insulin, the responses were less in frequency and magnitude, being 4 of 6 (2.7 ± 1.6), 3 of 11 (2.2 ± 1.1), and 0 of 12 (1.20 ± 0.55), respectively. The mean responses to islets of PBMCs from preclinical and clinical subjects differed significantly from control subjects ($P < 0.02$ by 2-tailed Kruskal-Wallis test). Secretion of granulocyte macrophage colony–stimulating factor by PBMCs over 6 days was assayed in the preclinical group and generally paralleled the uptake of [^{3}H]thymidine. PBMC reactivity to islets appeared to be at least as sensitive a marker of preclinical IDDM as autoantibodies to a 64,000-M_r protein, presumably the enzyme glutamic acid decarboxylase, in fetal pig proislets. In conclusion, islet-reactive T lymphocytes in subjects with preclinical and clinical IDDM can be identified in bulk culture of PBMCs. Detection of these autoreactive T lymphocytes should be of value in the diagnosis of preclinical IDDM and in monitoring the effects of immunotherapy. *Diabetes* 40:1128–33, 1991

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Received for publication 18 February 1991 and accepted in revised form 28 March 1991.

Insulin-dependent diabetes mellitus (IDDM) results from the autoimmune-mediated destruction of pancreatic islet β-cells (1). Evidence for the autoimmune basis of human IDDM includes infiltration of the islets by mononuclear cells (2–4) and the presence of circulating islet cell antibodies (ICAs; 5), insulin autoantibodies (IAAs; 6), and antibodies to a 64,000-M_r islet protein (64K), presumably glutamic acid decarboxylase (GAD; 7). These antibodies are markers of the preclinical stage of IDDM (1). In the spontaneous rodent models of IDDM, the NOD mouse (8) and BB rat (9), cell-transfer studies directly demonstrate the key role of T lymphocytes in mediating β-cell destruction. Evidence for the role of T lymphocytes in human IDDM is less direct but includes migration inhibition of blood leukocytes in the presence of islet homogenates (10); cytotoxicity of peripheral blood mononuclear cells (PBMCs) from IDDM subjects against human insulinoma cells (11); rat and human islets (12,13); inhibition by PBMCs of insulin release from mouse islets (14); generation from PBMCs of CD4 T-lymphocyte clones to human islet cells (15) or rat insulinoma membranes (16), one of which was shown to react to a 38,000-M_r insulin secretory granule protein (17); and reactivity to human insulin by PBMCs from subjects with preclinical IDDM (18). Some of these studies are not simple to perform or reproduce, and only one (18) has investigated preclinical IDDM, the stage in which T-lymphocyte reactivity to islet antigens should be maximal. We investigated whether subjects with preclinical and clinical IDDM could be identified by reactivity of their PBMCs to islets or insulin. Our aim was to develop a simple, direct test of T-lymphocyte reactivity to islet antigen(s) to facilitate the detection of subjects with preclinical IDDM and the monitoring of intervention therapy.

RESEARCH DESIGN AND METHODS

Experimental procedures were performed according to protocols approved by the Institutional Board of Medical Re-

TABLE 1
Clinical and experimental data for preclinical insulin-dependent diabetes mellitus (IDDM) subjects

Subject	Sex	Age (yr)	IVGTT (mU/L)	HLA-DR	ICAs (JDF U)	64K	IAAs (nU/ml)	Stimulation index of PBMCs to: Islets [^{3}H]thymidine	Islets GM-CSF	Human insulin [^{3}H]thymidine	Human insulin GM-CSF
1	F	33	142	3,4	640	++	110	4.3	12.0	5.10	6.6
2	F	16	183	3,4	40		9	11.0	20.0	1.00	1.0
3	M	9	31 (<5th percentile)	3,3	80	+	5	4.7	3.8	3.60	1.3
4	F	8	12 (<5th percentile)	ND	40	+	120	9.5	22.0	2.40	2.0
5	M	11	91	3,3	80	+	100	14.0	16.0	3.30	1.7
6	F	47	113	3,7	40	++	21	8.6	8.8	0.73	1.0
Mean ± SD								8.7 ± 3.7	13.8 ± 6.9	2.7 ± 1.6	2.3 ± 2.2

HLA-DR–matched human islets were used for subject 1; fetal pig proislets were used for subjects 2–6. IVGTT, intravenous glucose tolerance test (1 + 3-min plasma insulin); ICAs, islet cell antibodies; JDF U, Juvenile Diabetes Foundation units; 64K, antibodies to 64,000-*M*, islet protein (for grades, see METHODS); IAAs, insulin autoantibodies; PBMCs, peripheral blood mononuclear cells; GM-CSF, granulocyte macrophage colony–stimulating factor; ND, not done. The stimulation index is expressed as the ratio of stimulated to basal values. 64K band was graded visually as described in METHODS.

search and Human Ethics Committee. Subjects with preclinical IDDM (Table 1) were first-degree relatives of persons with IDDM and had a circulating ICA level ≥20 Juvenile Diabetes Foundation (JDF) U. They had an intravenous glucose tolerance test (19) in which the sum of the plasma insulin levels at 1 and 3 min after 0.5 g/kg i.v. glucose was used as the measure of first-phase insulin release. The range for the 1 + 3–min plasma insulin levels in 25 normal adults (mean age 31 yr) was 32–230 mU/L, with a mean of 95 mU/L and a median of 88 mU/L. Subjects with clinical IDDM (Table 2) met the diagnostic criteria for IDDM and were insulin dependent. Control subjects (Table 3), none of whom were positive for ICAs, IAAs, or 64K, were recruited from hospital and laboratory staff. When human islets were used to investigate the reactivity of PBMCs, subjects whose HLA types were known were selected to be HLA-DR identical to the islet donor. PBMCs from one or more control subjects were assayed in parallel with those from preclinical or clinical IDDM subjects.

Tissues were processed as follows. Human islets were isolated from pancreases (obtained with the consent of relatives) from organ-donor subjects by a ductal collagenase digestion technique as previously described (Collagenase type XI, Sigma, St. Louis, MO; 20). After Ficoll density-gradient centrifugation (Ficoll-Paque, Pharmacia, Uppsala, Sweden), islets were handpicked with a glass pipette under a dissection microscope and cultured (37°C, 5% CO_2 in air) free floating for 1–2 wk in RPMI-1640 medium containing 2% heat-inactivated neonatal calf serum. Fetal pig proislets, used because of the limited availability of human pancreases, were isolated by collagenase digestion of pancreases from late-gestation outbred fetal pigs obtained from a local abattoir, followed by culture in serum-free Dulbecco's modified Eagle's medium for 1–2 wk. Crude cell suspensions of

TABLE 2
Clinical and experimental data for clinical insulin-dependent diabetes mellitus (IDDM) subjects

Subject	Sex	Age (yr)	Duration of diabetes (yr)	HLA-DR	ICAs (JDF U)	64K	IAAs (nU/ml)	Stimulation index ([^{3}H]thymidine) of PBMC to: Islets	Human insulin
1	F	41	6.0	3,5	20	+		7.5	1.8
2	M	44	4.3	3,4				5.4	1.2
3	M	28	7.2	1,7				1.7	1.1
4	M	34	0.33	3,4	320		1900	11.0	1.6
5	F	58	>1.0	2,4	320	+	150	0.9	3.3
6	M	38	0.42	3,3	640	+++	1400	5.6	1.8
7	M	13	3.25	4,7	20	+		1.5	2.2
8	F	18	0.17	ND	80		15	6.2	
9	F	33	1.08	3,4	80		420	10.0	
10	M	12	0.04	ND	ND		120	2.1	2.5
11	F	12	0.08	3,4	ND	+	110	4.9	4.5
Mean ± SD								5.2 ± 3.4	2.2 ± 1.1

HLA-DR–matched human islets were used for subjects 1, 3, 5, and 7; fetal pig proislets were used for subjects 2, 4, 6, and 11. Note that subject 1 had polyglandular autoimmune disease. ICAs, islet cell antibodies; JDF U, Juvenile Diabetes Foundation units; 64K, antibodies to 64,000-*M*, islet protein (for grades, see METHODS); IAAs, insulin autoantibodies; PBMCs, peripheral blood mononuclear cells; ND, not done. The stimulation index is expressed as the ratio of stimulated to basal values. 64K band was graded visually as described in METHODS.

TABLE 3
Clinical and experimental data for control subjects

				Stimulation index of PBMCs to			
				Islets		Human insulin	
Subject	Sex	Age (yr)	HLA-DR	[^{3}H]thymidine	GM-CSF	[^{3}H]thymidine	GM-CSF
1	F	24	3,5	1.9	ND	0.92	ND
2	F	30	1,2	2.1	ND	0.94	ND
3	F	27	3,4	7.4	8.9	1.00	1.00
4	M	45	4,11	2.5	5.2	1.00	1.00
5	M	36	1,7	1.7	1.7	0.90	0.50
6	F	31	2,4	0.8	2.7	1.40	1.00
7	F	36	3,4	2.0	2.2	2.30	2.00
8	M	44	4,7	1.7	4.8	0.98	2.30
9	M	32	ND	2.2	3.5	0.74	0.94
10	F	38	ND	3.0	3.7	1.30	1.60
11	M	26	1,9	4.3	5.5	2.30	1.00
12	F	22	ND	3.0	5.8	0.71	1.30
Mean ± SD				2.7 ± 1.7	4.4 ± 2.1	1.20 ± 0.55	1.30 ± 0.55

HLA-DR–matched human islets were used for subjects 1, 2, and 5; fetal pig proislets were used for subjects 3 and 4 and 6–12. None of the subjects had 1st-degree relatives with autoimmune disease except for subject 3. PBMCs, peripheral blood mononuclear cells; GM-CSF, granulocyte macrophage colony–stimulating factor; ND, not done. The stimulation index is expressed as the ratio of stimulated to basal values.

fetal pig liver, kidney, and thyroid cells were prepared by incubating minced tissue in Hanks' balanced salt solution (HBSS) containing 2 mg/ml collagenase and 1.2 U/ml Dispase II (Boehringer Mannheim, Mannheim, Germany) for 20–30 min at 37°C, followed by washing in RPMI-1640/calf serum medium.

For measurement of PBMC reactivity, PBMCs were isolated from heparinized venous blood by centrifugation in a Ficoll-Hypaque gradient, washed twice with human tonicity RPMI-1640 medium, and adjusted to 2×10^6/ml with medium supplemented with 10^{-5} M 2-mercaptoethanol and 5% autologous serum. Autologous serum was used initially to approximate conditions in vivo but, after comparison with pooled human AB serum, was found to result in higher responses of PBMCs to islets. Heating at 56°C for 30 min to inactivate complement did not alter this effect. Whether the advantage of autologous serum is simply due to the fact that it is fresh or is specific, e.g., islet antibody related, has not been further investigated.

Three to 10 human islets or 5–15 pig proislets were handpicked and transferred in ~20 μl HBSS to round-bottomed wells in a 96-well plate (Linbro). Aliquots of 200 μl of PBMC suspension were added to quadruplicate wells containing islets or 5 μl of either 200 μg/ml human monocomponent insulin (Novo, Copenhagen) or anti-T-lymphocyte receptor monoclonal antibody OKT-3 (kindly provided by A. Boyd, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). Plates were incubated at 37°C in 5% CO_2 in air. On day 6, 50 μl of medium was removed from each quadruplicate well, pooled, and stored at −70°C for cytokine assays. [^{3}H]thymidine (1 μCi) was then added to each well, and after 14–18 h, the cells were harvested and counted in a liquid-scintillation spectrometer. Uptake of [^{3}H]thymidine into DNA was taken as the median of quadruplicate values, i.e., the mean of the two central values. .

Cytokines were measured in serial dilutions of conditioned medium: granulocyte macrophage colony–stimulating factor (GM-CSF) by the Insight GM enzyme-linked immunosorbent assay kit (Medical Resources, Sydney; lower limit of detection 35 pg/ml; intra- and interassay coefficients of variation [C.V.s] ~8 and ~14%), interferon-γ (IFN-γ) by the Human γ-Interferon Test EIA kit (Commonwealth Serum Labs, Melbourne; lower limit of detection 20 pg/ml, intra- and interassay C.V.s ~11 and ~16%), and tumor necrosis factor-α/β (TNF-α/β) by the mouse L929 fibroblast cytotoxicity bioassay (lower limit of detection 40 pg/ml; intra- and interassay C.V.s ~12 and ~21%).

ICAs were measured in serially diluted serum by indirect immunofluorescence on frozen sections of human organ-donor pancreas and expressed in JDF units relative to reference control sera. IAAs were measured by radioimmunoassay (normal range −15 to +39 nU insulin-bound/ml serum; intra- and interassay C.V.s at 100 nU/ml 5 and 15%; 21). 64K was detected by the ability of serum immunoglobulins prebound to protein G–Sepharose (Pharmacia, Piscataway, NJ) to precipitate a 64,000-M_r antigen from [^{35}S]methionine-labeled fetal pig proislets solubilized in 1% Triton X-100 buffer as previously described for human islets (22). The immune precipitates were subjected to sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis (SDS-PAGE) and autoradiography and the intensity of the 64,000-M_r band graded visually as +, ++, or +++. A band of the same size was precipitated from fetal pig proislets but not fresh pig liver or thymic cells by a sheep antiserum to purified rat brain GAD (23; provided by W. Blessing, Flinders Univ., South Australia) (Fig. 1).

The reactivity of PBMCs, measured as either counts per minute [^{3}H]thymidine uptake or nanograms per milliliter per 10^6 cells (per 6 days) cytokine, was expressed as the stimulation index (SI), i.e., the ratio of the stimulated to basal value. The significance of the difference between mean values was calculated by the nonparametric Kruskal-Wallis *H* test with two-tailed tables (24).

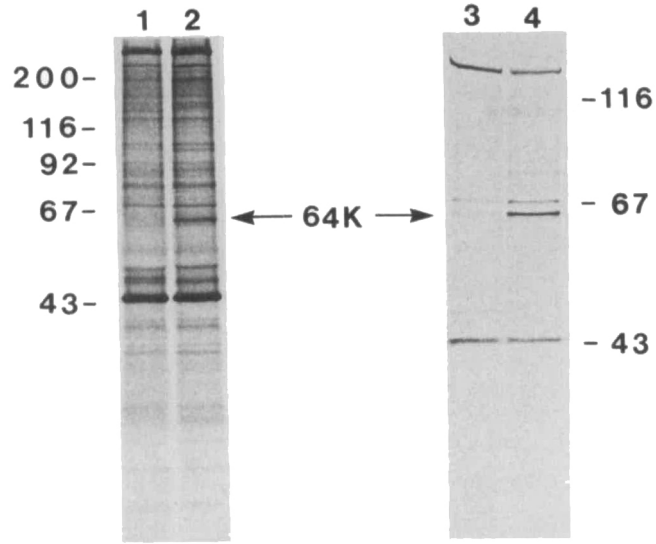

FIG. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography of immune precipitates from Triton X-100–solubilized [^{35}S]methionine-labeled fetal pig proislets. Immunoprecipitations were performed with 20 μl swollen protein G–Sepharose to which had been adsorbed serum from control subject (*lane 1*), clinical insulin-dependent diabetes mellitus (IDDM) subject 6 in Table 2 (*lane 2*), sheep immunized with type II collagen (*lane 3*), and sheep immunized with purified rat brain glutamic acid decarboxylase ([GAD] *lane 4*). *Arrows*, labeled 64,000-*M*, protein precipitated by IDDM serum (as doublet) and by sheep anti-GAD serum.

RESULTS

The response of PBMCs from preclinical and clinical IDDM subjects was related to the number of human islets or fetal pig proislets per well (for example, preclinical IDDM subject 6 in Fig. 2). Because of the limited supply of islets, an islet dose response could not be measured in each subject, and a standardized number of 10 fetal pig proislets or 5 adult human islets/well was used. The response to OKT-3, a positive control for T-lymphocyte stimulation, although generally exceeding that to islets, varied considerably, probably because OKT-3 was used in a soluble form and added only once at a submaximal concentration.

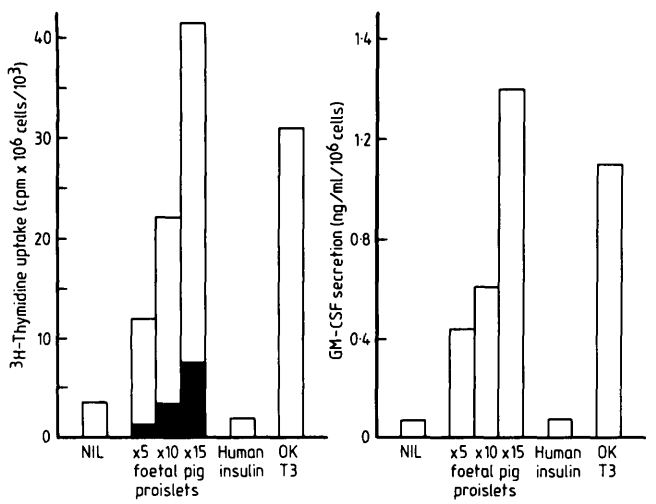

FIG. 2. Reactivity of peripheral blood mononuclear cells (PBMCs) from preclinical insulin-dependent diabetic subject 6 (Table 1) to fetal pig proislets, human insulin, and OKT-3. cpm, Counts per minute; NIL, PBMCs with no additives. PBMCs were incubated for 6 days, after which medium was removed for granulocyte macrophage colony–stimulating factor (GM-CSF) assay, and cells were pulsed with [^{3}H]thymidine (see METHODS for details). *Solid bars*, [^{3}H]thymidine uptake by islets in absence of PBMCs; in this condition GM-CSF secretion was not detected.

PBMCs from five subjects (clinical IDDM subjects 2, 4, and 9 and control subjects 4 and 7) were also cocultured with fetal pig liver, kidney, or thyroid cells (each ~1000, 10,000, and 25,000 cells/ml). Liver and kidney cells did not influence [^{3}H]thymidine uptake by PBMCs; at the highest concentration of thyroid cells, uptake by PBMCs from clinical IDDM subjects 4 and 9 and control subject 4 was stimulated but to less than half the level with proislets (data not shown).

The responses of PBMCs obtained from three subjects on three separate occasions over 10 wk are shown in Table 4.

The response of PBMCs to islets or insulin was regarded as significant if the SI of [^{3}H]thymidine uptake exceeded the mean + 2SD of the control group (Tables 1–3). The latter was calculated omitting control subject 3 because she was found to have three separate first-degree relatives with IDDM, Graves' disease, and multiple sclerosis, respectively, and her PBMC responses to pig proislets were consistently high (Table 4). On this basis, PBMCs from 6 of 6 preclinical, 7 of 11 clinical, and 1 of 12 control subjects reacted to islets, and PBMCs from 4 of 6 preclinical, 3 of 11 clinical, and 0 of 12 control subjects reacted to insulin. Although the SIs to islets or proislets are variable within groups (Tables 1–3), the differences between the group means for the preclinical or clinical subjects and the control subjects are highly significant ($P < 0.02$ by 2-tailed Kruskal-Wallis test; $H_t = 8.83$).

In addition to the subjects listed, we studied four females with Graves' disease and thyrotropin-receptor antibodies on treatment with carbimazole, two females and one male with diffuse scleroderma and antibodies to topoisomerase I, and one female with Sjögren's syndrome and antibodies to the ribonucleoproteins La and Ro. Only one of these subjects, with Graves' disease but no family history of IDDM or polyglandular autoimmune disease, had an elevated SI of [^{3}H]thymidine uptake to proislets of 5.5; the SIs of the other subjects were all ≤2.2.

GM-CSF, IFN-γ, and TNF-α/β were always detected in media from PBMCs incubated with OKT-3. GM-CSF was secreted from PBMCs that incorporated [^{3}H]thymidine in response to islets not from islets alone, but the SIs for GM-CSF secretion could be documented completely only for the preclinical group (Table 1) because conditioned media had been expended from most of the studies on the clinical subjects. In contrast to GM-CSF, TNF-α/β was never detected, and IFN-γ was detected inconsistently in media from PBMCs incubated with islets or insulin.

TABLE 4
[^{3}H]thymidine uptake by peripheral blood mononuclear cells

	Pig proislets	Human insulin	OKT-3
Clinical IDDM subject 4	11, 8.1, 9.8	1.6, 2.1, 1.4	16, 20
Control subject 3	7.4, 8.1, 6.0	1.0, 0.8, 1.0	3.8, 13, 10
Control subject 4	2.5, 3.5, 3.0	1.0, 1.0, 1.0	10, 5.5, 7.8

Table gives stimulation indices in 3 separate assays. IDDM, insulin-dependent diabetes mellitus.

DISCUSSION

Subjects with preclinical and clinical IDDM have T lymphocytes in their peripheral blood that exhibit increased reactivity to islet antigens. All preclinical subjects had a significant response to islets, including subject 2, who had undetectable IAAs or 64K. The responses in the clinical subjects were variable, but those who responded had a mean ± SD SI (7.2 ± 2.4) that was not significantly different from that in the preclinical group (8.7 ± 3.7). Four of seven clinical subjects whose PBMCs reacted to islets were 64K negative, whereas two of four clinical subjects whose PBMCs did not respond to islets were 64K positive. Although the sample sizes are small, these results suggest that reactivity of peripheral blood T lymphocytes to islet antigens is at least as sensitive as 64K as an index of anti-islet autoimmunity.

The presence of HLA-DR3 seems to denote the preclinical and clinical subjects whose PBMCs reacted to islets (Tables 1 and 2). Further studies are required to confirm this association and to demonstrate that it reflects MHC restriction of T-lymphocyte responses to islet antigen. The finding of islet-autoreactive T lymphocytes in control subject 3 with a strong family history of autoimmune disease and in one of four Graves' disease subjects tested emphasizes the need to control for disease and MHC. On the other hand, these two subjects, although lacking detectable antibodies to islet cell antigens, may have preclinical IDDM and are being followed up. Given the well-known clinical association between IDDM and Graves' disease, it would not be surprising to find islet-autoreactive T lymphocytes in some Graves' disease subjects and thyroid-autoreactive T lymphocytes in some IDDM subjects. We are undertaking further studies to elucidate whether the mechanism of this overlap relates to shared T-lymphocyte epitopes or to a common defect of immunoregulation.

Compared with islets, PBMC reactivity to human insulin in both preclinical and clinical subjects was lower and less frequent. This suggests that insulin, at least in soluble form, is not the major islet antigen eliciting the measured T-lymphocyte responses. This difference between islets and insulin is also observed with pork insulin and is unlikely to be insulin dose related because higher concentrations of insulin (10–25 μg/ml) actually inhibit responsive T lymphocytes (L.C.H., unpublished observations). Recent evidence indicates that the 64,000-M_r islet antigen in IDDM is the enzyme GAD in β-cells (7). GAD was identified in fetal pig proislets with a specific antiserum (Fig. 1). Nevertheless, absolute proof that GAD is responsible for the T-lymphocyte responses to fetal pig proislets or human islets will require purified or cloned GAD.*

Measurement of secreted cytokines may offer several advantages over [^{3}H]thymidine uptake in monitoring T-lymphocyte activation. In addition to functionally characterizing autoreactive T lymphocytes, the measurement of cytokines secreted over 6 days could provide an integrated index of T-lymphocyte activation independent of DNA synthesis and may avoid underestimating the latter if mitogenesis peaks before day 6. This argument is supported by the generally higher SIs obtained with GM-CSF compared with [^{3}H]-thymidine uptake in preclinical subjects, but further studies are needed to establish the sensitivity and reliability of measuring GM-CSF or other cytokines as indices of T-lymphocyte activation. Our inability to detect IFN-γ consistently or TNF-α/β at all might indicate that autoreactive anti-islet T lymphocytes detected by their response to exogenously added antigen are a functionally distinct subset, e.g., analogous to the TH2 CD4 cells of mice that secrete interleukins 4 and 5 in contrast to TH1 cells that secrete IFN-γ and TNF-β (26).

T-lymphocyte reactivity is likely to be the most relevant marker of islet autoimmunity and therefore should be useful for preclinical diagnosis and monitoring the response to intervention therapy. Furthermore, the presence of autoreactive anti-islet T lymphocytes in peripheral blood provides the basis for generating T-lymphocyte clones, which will facilitate our understanding of the pathogenetic mechanisms of human IDDM and the development of specific forms of immunotherapy.

*Since submission of this article, we molecularly cloned human brain and islet GAD (25), and preliminary studies demonstrated that PBMCs from IDDM subjects react to GAD.

ACKNOWLEDGMENTS

L.C.H. and I.L.C. were supported by the National Health and Medical Research Council of Australia. H.D.A. was the recipient of an Eli Lilly (Australia) Diabetes Fellowship.

The manuscript was critically reviewed by Dr. Margo Honeyman, and secretarial assistance was provided by Joyce Lygnos.

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