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Persistent T Cell Anergy in Human Type 1 Diabetes[1]

H.-Michael Dosch,[2]* Roy K. Cheung,* Wolfram Karges,[3]* Massimo Pietropaolo,[†] and Dorothy J. Becker[†]

An anergic phenotype has been observed in nonobese diabetic (NOD) mice and some autoreactive T cells from patients with type I diabetes. To better understand this phenomenon, we measured T cell proliferative responses to 10 diabetes-associated and up to 9 control Ags/peptides in 148 new diabetic children, 51 age- and MHC (DQ)-matched siblings (sibs), 31 patients with longstanding diabetes, and 40 healthy controls. Most (78–91%) patient and sib responses to glutamate decarboxylase of 65 kDa (GAD65), islet cell cytoplasmic autoantibody (ICA) 69, diabetes-associated T cell epitopes in ICA69 (Tep69), and heat shock protein (Hsp) 60 involved anergic T cells that required exogenous IL-2 to proliferate. Responses to proinsulin, IA-2 (and tetanus toxoid) required no IL-2 and generated sufficient cytokine to rescue anergic T cell responses. Most new patients (85%) had autoreactive T cells, three quarters targeting more than half of the diabetes Ags. Only 7.8% of the sibs and none of the controls had such multiple T cell autoreactivities, which thus characterize overt disease. Multiple anergic and nonanergic T cell autoreactivities were sustained during 2 yr follow-up after onset and in patients with longstanding (3–26 yr) diabetes. Activated patient T cells survived severe IL-2 deprivation, requiring 20–100 times less IL-2 than normal T cells to escape apoptosis. Diabetic T cell anergy thus persists for decades and is Ag and host specific but not related to disease course. Rescue by IL-2 from bystander T cells and high resistance to apoptosis may contribute to this persistence. These data explain some of the difficulties in the routine detection of disease-associated T cells, and they emphasize challenges for immunotherapy and islet transplantation. *The Journal of Immunology,* 1999, 163: 6933–6940.

T ype 1, or insulin-dependent diabetes mellitus (IDDM)[4] is a chronic, T cell-mediated autoimmune disorder of genetically susceptible hosts (1). Multiple diabetes risk genes contribute to the development of variable degrees of autoimmunity and overt disease (2, 3), but it remains uncertain how, when, and where they act or interact to modify normal host resistance toward a polygenic autoimmune trait.

β cell autoimmunity in humans and diabetes-prone rodents targets a similar spectrum of autoantigens that include insulin/proinsulin and several proteins of β cell and neuronal origin (1). These "diabetes-Ags" were defined in serological studies where the acquisition of multiple autoantibody specificities was strongly associated with progression of pre-diabetes to overt disease (e.g., Refs. 4–6). Less is known of the natural history of T cell autoreactivity (7), although patients with recent onset IDDM have autoreactive T cells that recognize glutamate decarboxylase of 65 kDa (GAD65)

(8–10), GAD67 (11–13), IA-2 (14, 15), insulin/proinsulin (16–18), islet cell cytoplasmic autoantibody (ICA) 69 (19–21), and other self Ags (8, 22, 23). However, the measurement of T cell autoreactivity in IDDM has overall been difficult and has not yet attained a level of clinical usefulness. Data presented here demonstrate that autoreactive T cells can be routinely measured and that diabetes onset is characterized by multiple T cell autoreactivities as well as multiple autoantibodies.

We previously developed a serum-free assay system for proliferative T cell responses that allowed detection of autoreactive T cells undergoing anergy upon cognate activation, characterized by normal induction of IL-2 receptor expression but insufficient IL-2 production (20, 24). Here we asked how common are such cells, what is their Ag specificity, and how do they relate to disease course before, at, and after onset of overt IDDM. Based on lengthy pilot studies (24, 25), because of the small response amplitudes routinely observed, and as a basis for a long-term prospective study of pre-diabetes progression, we designed a large, blinded study protocol with stringent validation criteria for the assay system.

Our assay system could detect T cells autoreactive to all of the 10 diabetes-relevant test Ags employed here, although prevalences of positive responses differed. Diabetes onset was characterized by multiple autoreactivities, which were very rare in MHC-matched siblings (sibs) and absent in healthy controls. In patients and the smaller subset of sibs that had autoreactive T cells, most responses to heat shock protein (HSP) 60, GAD65, ICA69, diabetes-associated T cell epitope in ICA69 (Tep69), BSA, and diabetes-associated T cell epitope in BSA (ABBOS) were anergic and required exogenous IL-2. In contrast, T cells specific for proinsulin, IA2, and tetanus toxoid were not anergic. Diabetic children maintained anergic and nonanergic T cell autoimmunity over a 2-yr prospective follow-up period, and patients with longstanding IDDM (3–26 yr) still maintained multiple anergic and nonanergic T cell pools. T cell anergy may contribute to difficulties in their routine detection. We document a greatly enhanced resistance to IL-2 deprivation and apoptosis

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[4] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; ABBOS, diabetes-associated T cell epitope in BSA (see Table II); FDR, first degree relative of index case with IDD; GAD65, glutamate decarboxylase of 65 kDa; ICA, islet cell cytoplasmic autoantibodies; PI, proinsulin; RR, relative risk; SI, stimulation index (mean cpm test:mean cpm control); Tep69, diabetes-associated T cell epitope in ICA69 (see Table II); sibs, siblings; HSP, heat shock protein; NOD, nonobese diabetic.

Table I. *DQB alleles in patients and sibs*

Allele	Sibs (%)	Patients (%)	p
DQB1*0201/0302	26	37	0.47
DQB1*0302/not 201	26	19	0.57
DQB1*0201/not 302	30	34	>0.9
DQB1*0602/x	5	2	>0.9
Other	13	8	0.36

in patient T cells that may contribute to the persistence of anergic T cell pools and autoreactive T cells in general.

Materials and Methods

Study populations

Consecutive patients with IDDM, diagnosed at the Children's Hospital of Pittsburgh ($n = 148$, age 10 ± 4.18, range 1–18 years, 142 white, 6 black) were recruited with informed consent. Two thirds were analyzed in the first week of diagnosis and the remainder within 2 mo. Subsequently, 109 index cases provided 2.8 ± 0.8 (range 2–5) consecutive follow-up samples for 22 mo after onset. Siblings of index cases ($n = 51$) were matched for age (age 10.6 ± 4.29, range 1–18, $p = 0.35$ vs patient ages) and recruited into a control cohort. These siblings were chosen to provide age- and MHC-matched controls that shared environment and a proportion of the autoimmune predisposing genes with patients, but have a long term disease risk of only 5–10% (26, 27). As shown in Table I, siblings and patients had similar MHC class II (DQ) alleles. Five siblings had GAD65- and five had had ICA, but no risk-associated DQ alleles. Patients with longstanding IDDM were recruited from Registry records ($n = 31$, age 19.2 ± 11.6 yr, disease duration 9.2 ± 5.1, range 3–26 yr, age at onset 9.4 ± 5.2 yr). Forty unrelated, healthy volunteers with no family history of diabetes were tested on one to four occasions (age 40 ± 9 yr, range 19–55).

Experimental procedures

PCR-based DQ tissue typing followed standard procedures (26, 28, 29). ICA were measured by indirect immunohistochemistry on cryopreserved human organ donor as well as rat pancreas sections (26). The measurement of GAD65 and IA-2 autoantibodies employed standard liquid phase radio-immunoassays (5), and insulin autoantibodies were measured with the Kronus kit (Kronus, Sheffield, U.K.). These assays have shown excellent sensitivity and specificity in multiple proficiency workshops.

Blood samples for T cell assays received preservative-free heparin and were shipped to Toronto by overnight courier in Styrofoam isolators without ice. Until reaching an enrollment of 92 new onset patients (62% of the study population), the status of sample donors (patient, sib, healthy control)

was blinded to the Toronto lab. To test intraassay variability, samples from 12 subjects were split in Pittsburgh and shipped with different identifications ("blind duplicates series"). Samples usually had a cell viability of ≥95%. Blood samples from 22 newly diagnosed Toronto patients and 33 of their first degree relatives were analyzed fresh and after overnight storage at room temperature. We previously reported that neither prevalences of positive T cell responses (p values ≥0.3, Fisher's exact test) nor mean amplitudes of responses to the various test Ags were different between Pittsburgh and Toronto patients with or without overnight storage ($p > 0.2$) (25).

T cell proliferation assay

Mononuclear cells were enriched on Ficoll-Hypaque gradients, and 1×10^5 cells per flat-bottom microculture well were incubated in 200 μl serum-free Hybrimax 2897 medium (Sigma, St. Louis, MO) with or without 0.005–10 μg/ml of the test Ags (Table II). Ags (50 μl in medium) were added to replicate dry wells and allowed to adhere before addition of other culture ingredients and cells. Optimal Ag doses were reasonably consistent from responder to responder, and we omitted full Ag dose responses when cell yields were limiting. To detect anergic T cells, parallel sets of cultures received 10 U recombinant human IL-2 in addition to test Ags, as previously described (20). Unless indicated otherwise, we will refer to results from IL-2-supplemented responses throughout this paper. After 6 days, cultures were pulsed overnight with 1 μCi [^{3}H]thymidine, harvested, and submitted to scintillation counting. Data are presented as average cpm or mean stimulation indices (SI, cpm test ÷ cpm unstimulated culture) (20). Addition of IL-2 raised background [^{3}H]TdR incorporation on average 2-fold, thus lowering SIs by that factor.

To analyze T cell survival, patient, sib and control cells were stimulated with anti-CD3 (0.5 μg/ml; Calbiochem, La Jolla, CA). Replicate test cultures received 0–100 μg anti-IL-2 (R&D Systems, Minneapolis, MN). Cell death was quantified 3 days later using BCECF as described (30); cell survival was measured through [^{3}H]TdR incorporation in parallel cultures.

T cell test Ags

Eleven diabetes-associated and control Ags/peptides were routinely used (* in Table II). In addition, three self and five control Ags/peptides were tested in subsets of study subjects as discussed in the text. All data on HSP60 were obtained with peptide p 227 (31). In pilot studies, two *Escherichia coli*-expressed preparations of GAD65 gave unacceptable variability and considerable numbers of positive responses in healthy controls. Based on pilot data (24) as well as seven published reports (10, 32–37), we used GAD65 peptide 524 –543, and all GAD65 data presented were obtained with this peptide. A baculovirus-expressed preparation of GAD65 has since become available, and 18 of 20 GAD65–524-reactive patients showed positive, anergic responses to the protein as well.

Table II. *Test Ags[a]*

Ag	Description/Source	Derivation, Comments
PI	Lilly Pharm.	*E. coli,* full-length*
IA-2	Bayer Pharm.	*E. coli,* "ICA512"*
GAD65	Peptide 524	SRLSKVAPVIKARMMEYGTT*
HSP60	Peptide 277	VLGGGCALLRCIPALDSLTPANED*
ICA69	β-isoform	*E. coli* *
	α-isoform	Baculovirus[†]
	ICA69-36 "Tep69"	AFI**KATGKK**EDE, homolog BSA-148*
	ICA69-202	KN**FDKLK**MDVCQ, homolog BSA-394[†]
	ICA69-350	SEE**GACL**GP**V**AG, homolog BSA-193[†]
BSA	Sigma	Fraction V*
	BSA-148 "ABBOS"	FKADE**KK**FWGKYL, homolog ICA69-36*
	BSA-394	TSV**FDKLK**HLVD, homolog ICA69-202[†]
	BSA-193	EDK**GACL**LPKIE, homolog ICA69-350[†]
Tetanus toxoid	PMC Canada	Pharmaceutical grade*
OVA	Sigma	Fraction V*
	OVA152	EYQDNRVSFLGHFI*
Human hemoglob.	Sigma	Purified[†]
Human cytochr. *c*	Sigma	Purified[†]
Human actin	Sigma	Purified[†]

[a] Description of the test antigens/peptides used, not all could be tested in all study subjects. Eleven Ags (marked with an asterisk) were used routinely; the remainder (marked with a dagger) were tested only in subsets of probands to answer specific questions. Peptide names indicate origin and N-terminal amino acid position. Residues printed in bold identify homologies described in the text.

Data analysis and statistics

Our T cell assay validation criteria defined that 1) responses should show consistent Ag dose kinetics, 2) blinded and unblinded data sets should delineate similar distributions (e.g. % positives/negatives) in the different cohorts, and 3) the blind duplicate series should show less than 20% intraassay variation. In addition, and similar to the practice in IDDM autoimmune serology (e.g., Ref. 38), our main functional validation criterion required that a positive/negative discriminator allow distinction of patient from control cohorts. OVA and/or OVA peptide p157 were used as negative control Ag/peptide. Proliferative responses to a given Ag/peptide with a stimulation index 4 SDs above mean OVA/OVA157 peptide responses were deemed positive. Proliferation in OVA- or OVA157-stimulated cultures was similar to that in unstimulated cultures ($p > 0.2$). Tetanus toxoid provided a control Ag expected to give positive responses in the majority of samples.

Mann-Whitney tests were used to compare numeric results. Significance was set at 5%, all p values were two-tailed. Since data distribution was often skewed, nonparametric regression data are reported where applicable (Spearman, corrected for ties). Fisher's exact test was employed to analyze tables, using Katz' approximation to calculate relative risks (RR). For larger tables, we performed χ^2 tests with Yates correction.

Results

T cell assay validation

Results from well over nine thousand replicate T cell assays were evaluated in this report. Thymidine incorporation in cultures with positive responses showed similar replicate variation within $\pm$ 11% of the mean. This figure is within the validation criteria and suggests that the numbers of islet reactive T cells was not limiting (39).
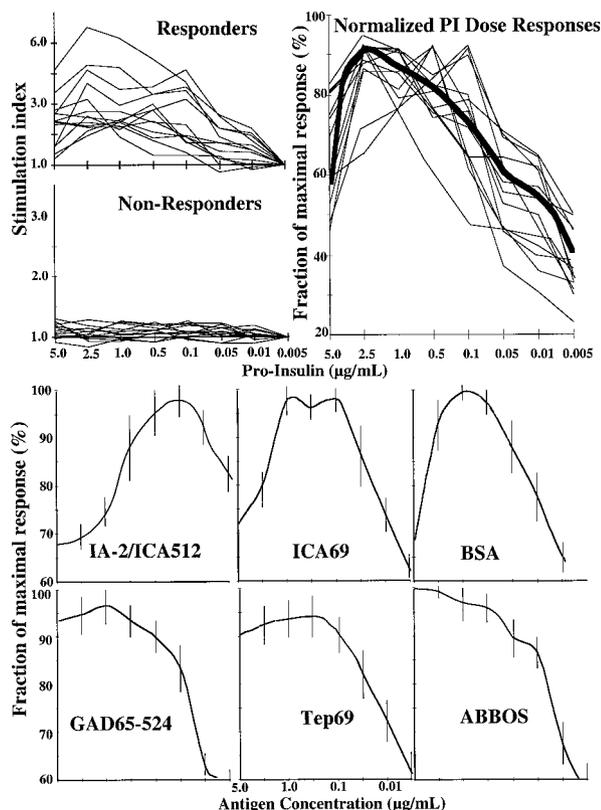

FIGURE 2. Blind duplicate series. Blood samples from 7 patients and 5 FDRs were split into two aliquots, each labeled with a different ID and sent to the Toronto assay lab without announcement. Each sample gave 96 replicate responses to the various test and control Ags. Data (mean cpm [^{3}H]TdR) from set 1 and 2 are plotted on x- and y-axes, respectively; many points overlap.

Positive T cell responses to diabetes-associated Ags/peptides followed distinct Ag dose kinetics (Fig. 1). Reasons for our choice of proinsulin over insulin as test Ag included results of pilot experiments where insulin often showed erratic, U-shaped dose responses in patients as well as some controls (not shown). Diabetes-associated test Ags/peptides (Fig. 1) had defined dose optima, usually between 0.1–1 μg/ml. Since responders to HSP60 were rare, a full dose-response curve was available from only four HSP60-reactive patients (not shown). Fig. 1 (*top panel*) also illustrates, with proinsulin as an example, that responders and nonresponders were clearly distinguished. On a molar basis, insolubilized protein Ags were processed and presented with 100-fold higher efficiency than peptides (see *Materials and Methods*). There were no positive responses to human hemoglobin, cytochrome c, actin, or four control peptides derived from regions of homology between ICA69 and BSA outside of the Tep69/ABBOS epitopes (Table II) (20, 21).

After unblinding (see *Materials and Methods*), samples were identified according to diabetes/control status to allow additional T cell function studies (see below). The prevalence and amplitudes of positive responses in blinded and unblinded study parts were similar (p values >0.2). Split samples from 12 subjects were analyzed in a blind duplicate series (Fig. 2). This generated two sets of 96 replicate assays/set. Mean response amplitudes in the sample pairs were highly correlated ($r = 0.97$, $p < 0.0001$), and the intraassay variation was $\pm$ 6.7%, well within the validation criteria.

Responses to tetanus toxoid, observed in 96% of samples, were larger than those to any of the other test Ags ($p < 0.0001$), and there was no OVA response in this data set (Table III). The prevalence of positive tetanus responses was similar in all three groups, but healthy controls had higher mean tetanus response amplitudes than patients or sibs ($p < 0.001$). Positive responses to each of the diabetes Ags were far more frequent in patients than in siblings ($p < 0.0001$, Fig. 3), which in turn had more positive responses than healthy population controls ($p = 0.0009$). Responses to the diabetes Ags were rare in healthy volunteers: seven had self-reactive T cells recognizing a single islet Ag. In addition, there were some positive responses to the one environmental Ag used, BSA ($n = 4$) or its ABBOS epitope ($n = 1$) (20). Normal controls were

FIGURE 1. Dose response kinetics for a given test Ag were initially established in at least 20 patients each. Proinsulin was added (*top panels*) in the concentrations indicated to cultures of PBMC from 24 patients; 13 of the 24 showed positive (*upper left*) and 11 no responses (*lower left*). Positive responses were normalized by calculating each data point as a percentage of maximum for a given donor (*upper right panel*, heavy line: group mean). Responses (*lower panels)* to IA-2, ICA69, BSA, and the GAD65, Tep69, and ABBOS peptides were similarly normalized and plotted.
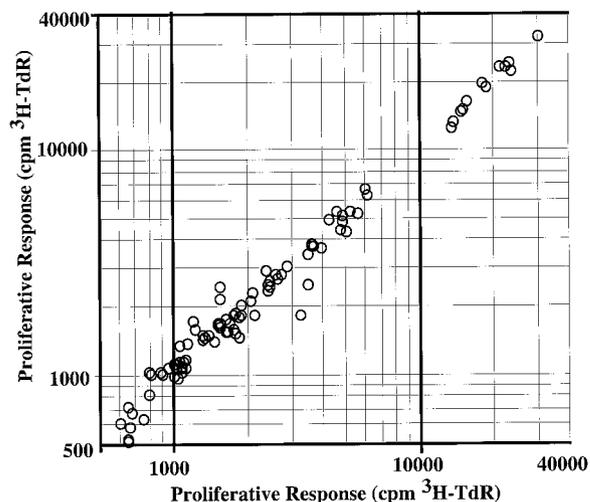
Table III. *T cell responses in different cohorts*[a]

Ag	Normals (%) (n = 40)	Low Risk Sibs (%) (n = 51)	IDDM (%) (n = 148)	Prevalence IDDM vs Sibs
Tetanus toxoid	90.3 (7.8 ± 1.9)	100 (5.4 ± 1.6)	93.8 (5.8 ± 2.1)	p = 0.63
PI	2.4	35 (2.3 ± 0.67)	79 (2.7 ± 1.36)	p < 0.0001
IA-2/ICA512	0	32 (3.1 ± 0.76)	78 (2.1 ± 0.44)	p < 0.0001
ICA69	4.9 (1.8 ± 0.16)	29 (1.9 ± 0.25)	62 (2.3 ± 0.54)	p < 0.0001
Tep69	0	22 (1.9 ± 0.37)	51 (2 ± 0.62)	p < 0.0001
BSA	9.6 (1.9 ± 0.6)	16 (1.7 ± 0.29)	55 (2 ± 0.51)	p < 0.0001
ABBOS	2.4	24 (1.9 ± 0.31)	62 (2.1 ± 0.54)	p < 0.0001
GAD65	4.9 (2.3 ± 1.12)	31 (1.9 ± 0.39)	55 (2.3 ± 0.61)	p < 0.0001
HSP60	2.4	8 (1.9 ± 0.43)	16 (2.1 ± 1)	0.09
OVA	0	0	0	
Other controls[b]	0	0	0	

[a] Prevalence (%) and amplitudes (mean ± SD) of positive proliferative responses to test Ags.
[b] Other control Ags (human hemoglobin, cytochrome *c,* actin) were tested only in 9 normals, 14 sibs, and 27–36 patients.

reexamined one to four times over the ensuing 22 mo, and only two positive responses remained, whereas three new responses appeared transiently. This contrasts with results from the patient follow-up study, which indicate that response patterns are stable within 2 yr of prospective follow-up (see below).

The amplitudes of positive responses to diabetes Ags were small, with mean SI values around 2 (Table III). Nevertheless, definition of positive responses as 4 SDs above background proliferation delineated significant differences in the prevalence of positive responses to a given test Ag between patients, low risk sibs, and population controls (Fig. 3, Table III). Overall, the assay system appears to be valid by our criteria. T cell pools recognizing diabetes-relevant Ags 1) circulate in peripheral blood of most diabetic individuals, 2) are detectable in some of their relatives, but 3) rarely and transiently in healthy population controls.

Effects of exogenous IL-2

Most (78–91%) positive responses to GAD65, ICA69, Tep69, BSA, ABBOS, and HSP60 Ags/peptides were observed only in the presence of added IL-2, consistent with the presence of anergy

(Fig. 4). In contrast, responses to proinsulin, IA-2, and tetanus toxoid showed essentially no IL-2 effect (Fig. 4). The remaining 10 test Ags/peptides (Table II) failed to elicit responses in the presence or absence of added IL-2. Anergic and nonanergic response patterns were the same in patients and sibs (including those from fresh Toronto samples reported earlier (24, 25)). Anergic responses were thus Ag, but not donor or disease course specific, and they covered several but not all diabetes Ags even in subjects with a low risk to develop the disease. This is reminiscent of NOD mice where a broad tendency to sustain anergic T cell pools has been described (40).

The co-existence of anergic and nonanergic T cells that target diabetes Ags may have mechanistic implications. As shown in Table IV, anergic responses could be rescued not only by addition of IL-2, but also by costimulation with either proinsulin, IA2, or tetanus. Proliferation in cultures stimulated by OVA or with other "anergic" Ags showed no rescue effect. Thus, in a host with both anergic and nonanergic T cells, the latter may rescue the former if they happen to encounter their respective Ag at similar times and locales, such as a given islet. However, rescue can also be provided

FIGURE 3. T cell responses (mean SI) to diabetes-related test Ags in newly diagnosed patients (n = 148), age- and MHC class II (DQ)-matched siblings of index cases with a low risk of developing diabetes (n = 51), and in unrelated, healthy general population controls (n = 40).
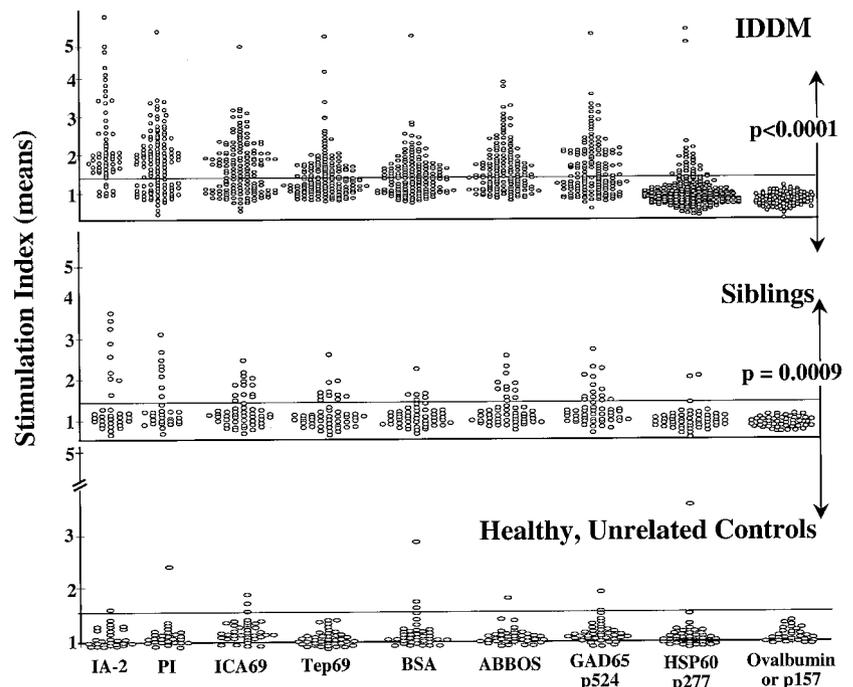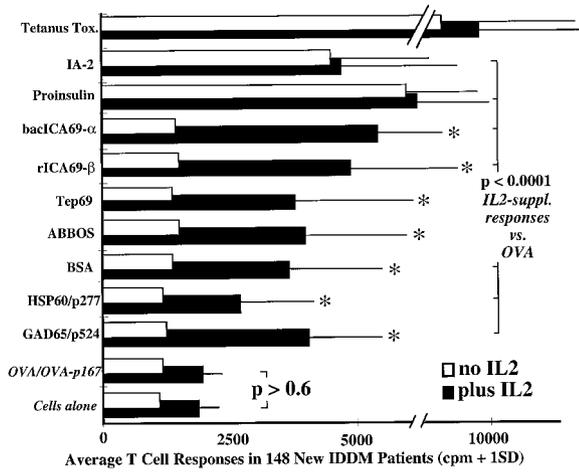
FIGURE 4. T cell responses to the Ags indicated in 148 patients with recent onset IDDM. Data are presented as overall means + 1 SD, the latter reflecting mainly the distribution of response amplitudes in this cohort. White bars, no IL-2 added; black bars, IL-2 added. ∗, IL-2 effect; $p <$ 0.0001.

by other bystanders, not primarily related to diabetes, in this case tetanus toxoid-activated T cells (Table IV). Bystander mechanisms have previously been implied in diabetes development (41).

Autoimmunity at disease onset

As the main control cohort for patient data analysis, we used siblings with a low risk to develop diabetes, matched for MHC (Table I), age, and environment. Except for tetanus, OVA, and HSP60, patients had T cell responses to the test Ags in 85% (Table III), three quarters targeting multiple (at least four) test Ags. Since 82% of patients had multiple ($\geq$3) autoantibodies, no correlation could be delineated between any autoantibody and any (anergic or nonanergic) T cell responses measured. In contrast, only four siblings (7.8%) responded to multiple diabetes Ags (Fig. 3), whereas two thirds had no or one positive T cell response (p values <0.0001, Table III). None of the healthy controls had multiple T cell responses.

Table IV. *Rescue of anergic T cells*[a]

	Medium	ICA69	Tep69	GAD65	OVA
IDDM					
Medium	1129				
IL2	2436	5572	5103	4534	2216
ICA69	1382	1171			
Tep69	1440	1420	1295		
GAD65	1479	1268	1266	1184	
OVA	1289	1098	1306	1240	1105
PI	2980	9431	8181	7860	3113
IA2	3244	7864	7717	7389	3052
Tetanus	5879	12204	11640	11137	5412
Sib					
Medium	1224				
IL2	1998	2403	2218	4584	2149
ICA69	1382	1234			
Tep69	1440	1186	1261		
GAD65	1379	1322	1468	1244	
OVA	1088	1077	1209	1310	1187
PI	1330	1573	1410	1461	1259
IA2	1442	1242	1365	1348	1086
Tetanus	6430	6653	6591	8927	6317

[a] Rescue of anergic T cells by costimulation with the "non-anergic" diabetes-associated Ags, PI or IA2 or with tetanus toxoid. Mean thymidine incorporation (cpm) in cultures stimulated as indicated. Redundant entries are blank. SD were within ±10%. Thirteen additional patients and four sibs were tested with similar results.

Table V. *Mimicry in diabetics and sibs*[a]

	No. of Patients (%)	No. of Sibs (%)
ABBOS⁺/ICA69⁺	91 (99)	9 (64)
ABBOS⁺/ICA69⁻	1 (1)	5 (36)

[a] Comparison of patients and siblings with positive responses to the ABBOS mimicry epitope in BSA. $p = 0.0001$; RR = 32.8.

We were able to compare several peptides and isoforms of one autoantigen, ICA69. Patient responses to the full-length α-, the C-terminal truncated β isoforms of ICA69, and to Tep69 peptide (21) were concordant ($r = 0.84$, data not shown), suggesting the absence of important target epitopes outside of Tep69. We have previously suggested that mimicry between an epitope present in dietary cow milk and ICA69 may play a role in diabetic autoimmunity and demonstrated, in both patients and NOD mice, T cells that show antigenic mimicry between the BSA-derived ABBOS peptide and its ICA69 homologue, Tep69 (20, 21, 42). In the present study, responses to ABBOS and ICA69/Tep69 were highly concordant in patients, but not low risk siblings (Table V, $p = 0.001$, RR = 32.8). Mimicry thus appears to be a marker of disease risk, despite the fact that these cells are anergic by our criteria. To test this possibility, we have begun to analyze first degree relatives of our index cases that have a high risk to develop overt disease, based on the presence of autoantibodies and risk-associated but not protective DQ alleles (26, 27). Only 1 of 27 tested high risk subjects with Tep69-reactive T cells had undetectable ABBOS responses ($p = 0.012$ vs sibs, $p = 0.4$ vs patients). The maintenance of these cross-reactive T cell pools thus is associated with progressive pre-diabetes and high disease risk.

Diabetes onset is thus characterized by the presence of multiple autoantibodies and T cells that target multiple diabetes Ags. Overall, autoreactive T cells were significantly associated with disease (patients), and with familial diabetes disposition (sibs). In none of the cellular or humoral immune parameters analyzed here did we discern a significant relationship between age or sex. A tendency for more T cell responses in patients with high onset levels of hemoglobin A1c did not reach significance ($r = 0.18$, $p = 0.06$). This data set indicates that anergic and nonanergic T cell autoreactivity is Ag specific and common at disease onset, but present also in a subset of sibs, few of whom are expected to develop diabetes.

Autoimmunity after diabetes onset

Blood samples ($n = 297$) from 109 index cases were analyzed on two to five occasions over a period of 22 mo after onset (Fig. 5A). In 64.2% of these patients, positive and negative response patterns to a given test Ag remained the same throughout the observation period, 17% showed additional responses to one (10%) or more (7%) test Ags, and 13% lost reactivity to one or more. Only two patients (1.8%) had very variable patterns, and five showed intermittent changes that returned to onset patterns. A trend for lower prevalences of T cell responses early after onset did not reach significance ($p > 0.2$). The fact that proinsulin responses were at best intermittently affected by insulin therapy would tend to confirm reports that target epitope(s) lie outside of the mature insulin molecule (43). Anergic T cells did not behave differently from nonanergic T cells recognizing proinsulin or IA2.

As a comparison group, we analyzed 31 patients with longstanding IDDM (3–26 yr) (Fig. 5B). Surprisingly, 35% recognized almost all test Ags, 54% recognized half or more, and only 16% had no or one responses. Humoral autoimmunity was active as well, with 63% of patients still positive for ICA. For example, a patient
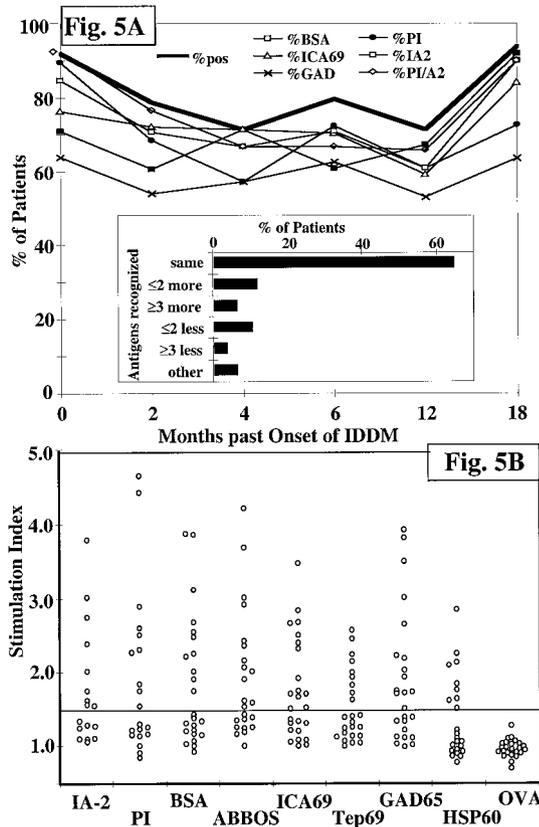
FIGURE 5. Persistence of T cell autoreactivity past onset of IDDM. *A,* Of the study index cases, 109 were followed prospectively for T cell responses every 2–4 mo. The prevalence (%) of positive responses is shown. *Insert,* Stability of T cell responses in each patient. Two thirds show the same response patterns throughout the follow-up period; some gain or lose one or more responses. *B,* T cell responses (SI) in 31 patients with long-standing (9.2 ± 5.1, range 3–26 yr) IDDM. Not all responses could be measured in all patients.

FIGURE 6. Apoptosis resistance in patients, siblings, and controls. Lymphocytes were activated with anti-CD3 Ab, and 0–100 μg/ml of anti-IL-2 was added. The graph indicates the amount of IL-2 required for a 50% reduction in cell survival. Cell death was measured through BCECF catalysis. [³H]TdR incorporation gave similar results in parallel cultures.

with 22 years disease duration and undetectable levels of C-peptide for many years, had ICA of 320 JDF units and T cells responding to all test Ags but HSP60. There was no correlation between humoral or cellular autoreactivities or the duration of disease. Anergic and nonanergic T cell pools again behaved similarly, suggesting that T cell anergy was independent of disease status and duration.

Abnormalities of diabetic T cells

While the basis of anergy to some of the Ags measured here is unknown, the reversal by IL-2 indicates that IL-2 deprivation plays a critical role. The appearance of anergic T cells in even young siblings with low disease risk and minimal signs of autoimmunity, and the persistence of these cells even decades after disease onset, implied the possibility of an underlying abnormality in T cell function, as has been demonstrated in nonobese diabetic (NOD) mice (e.g., Refs. 40, 44, 45). Lymphocytes from patients, sibs, or controls were stimulated with anti-CD3 in the presence or absence of graded amounts of anti-IL-2. When measured in an apoptosis assay, anti-IL-2 induced cell death in a dose-dependent fashion as expected (not shown). However (Fig. 6), patient and some of the sib T cells required dramatically (20- to 100-fold) higher anti-IL-2 doses than controls. Measuring [³H]TdR incorporation gave the same results (not shown). These data indicate that diabetic T cells can survive even severe IL-2 deprivation, which kills activated normal T cells. In this sense, T cell function in diabetes is highly IL-2
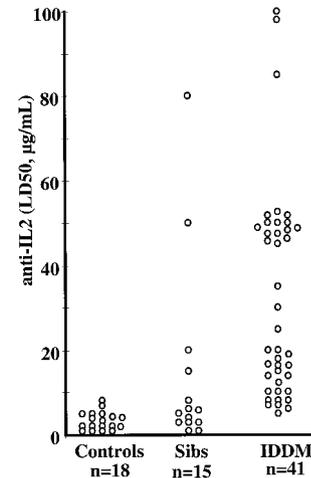
sensitive, enhancing susceptibility to bystander effects and synergizing with the reluctance to die toward persistence of anergic cells.

Discussion

This large data set provided validation for the small T cell proliferative responses to diabetes-associated Ags observed in this study and possibly explains difficulties in the routine detection of several such responses with anergy. Confirming and extending two smaller, previous studies (14, 22), multiple T cell autoreactivities, anergic as well as nonanergic, characterize overt disease. This should not come as a surprise, considering that multiple autoantibodies also appear near disease onset. The demonstrated bystander effects and resistance to apoptotic T cell ablation are good candidates for factors that contribute to the long persistence of β cell autoimmunity.

Assay validation was an important part of this study, and we conclude that all validation criteria were met. The stability of T cell response patterns in index cases over the 2 yr of prospective follow-up support this conclusion.

Previously reported observations and pilot experiments for the present study established that the responses measured here involved MHC class II-restricted CD4$^+$ T cells that respond to cognate activation with IL-2 receptor expression and recruitment of p56^{lck} (20, 25, 46, 47). The absolute response amplitudes observed were small, as has been observed by others (13, 16). In preliminary experiments, we also measured cytokine production in proinsulin- or IA2-stimulated cultures. IL-4, IL-10 and IFN-γ responses were found in 5 of 11 patients studied, but the ratios of IFN-γ, IL-4, and/or IL-10 varied. Two patients had neither proliferative nor cytokine responses, and four produced mainly IFN-γ. Further cytokine studies could be of mechanistic interest and help to distinguish relatives with high or low disease risk.

Our definition of positive responses was based on proliferation in cultures containing a control protein or peptide OVA/OVA157. Using thymidine incorporation either in unstimulated cultures or in MHC-matched siblings or in unrelated controls unresponsive to the test Ags would not have altered the principal study conclusions but added variability. Thus, the data analysis strategy was optimal to distinguish T cell responses of patients, low risk sibs, and controls. This is a practice commonly used to validate autoantibody

assays (e.g., Refs. 38 and 48). Our definition of positive responses as an SI $\geq$ 4 SDs above control values has statistical power and accommodates intraassay variation; hard cut-off rules for SI values such as 2 or 3 are arbitrary and would have reduced the strength of patient-sib distinctions.

In our hands, a serum-free culture system appears to exaggerate or unmask the requirement for exogenous IL-2 in responses to some diabetes Ags. Among published reports of GAD65, ICA69, or HSP60 responses (8–13, 19), only ours used a serum-free culture system and identified anergic T cells (20, 21, 46). Serum supplements may in some way contribute to responses observed, perhaps allowing low level IL-2 production by recently activated, circulating bystander T cells that rescues some anergic lymphocytes; the IL-2 deprivation studies emphasize how little IL-2 is needed by patient T cells.

The Ag restriction of anergy was robust. It will be necessary to determine whether these Ags activate T cells in a peculiar way, perhaps through unusual MHC binding (49, 50), which is biased toward anergy in a precarious fashion (51). Inherent T cell abnormalities may contribute to anergic responses; signaling abnormalities have been associated with abnormal anergy in NOD mice (52).

Where tested, protein and peptide (epitope) responses followed the same patterns. This could imply that anergic T cell responses reflect peculiarities of the sensitization process to these Ags, which in the case of the BSA/ABBOS↔ICA69/Tep69 mimicry pairs of Ags includes exposure through the oral route (53, 54). We recently tested a full-length GAD65 preparation and found that responses nearly always (18 of 20 responders) required exogenous IL-2, like the GAD-524 peptide used throughout the study. Responses to the study GAD peptide thus are likely typical for responses to GAD65 in general. Preliminary data from a collaborative study with A. Notkins (National Institutes of Health, Bethesda, MD) identified several peptide target epitopes in IA2, and all these responses were nonanergic (A Notkins, unpublished observations).

Autoimmunity continues unabated in many patients long after disease onset. This confirms clinical observations (55–57) and presents a challenge for transplantation and gene therapy approaches in diabetes. Humoral autoimmunity was previously reported to continue in C-peptide-negative patients (58, 59). It is unclear what sustains this autoimmunity. Relevant islet Ags might be supplied through continued regeneration of β cell precursors, sufficient to maintain autoimmune repertoires, even if these β cells die before functional maturity. If correct, then immune intervention even at a late stage of disease could have promise if it was effective.

We were surprised that anergic T cells persisted as well and as long as nonanergic cells. Our observations delineate two mechanisms that could contribute to persistence: an ability to resist cell death even under severe IL-2 deprivation and rescue from apoptotic ablation through IL-2 from bystander lymphocytes. The molecular mechanisms of death resistance are unknown, but abnormal expression of members of the bcl-2 family of proteins has been described in NOD mice (44, 45) and needs to be examined in patient T cells, as does the role of other cytokines, such as Type I IFNs (60). High resistance of autoreactive T cells to cell death may explain the difficulties to break autoimmunity through immunosuppressive therapies (59).

In an extension of these studies to a large cohort of first degree relatives with varying levels of diabetes risk (H.-M. Dosch et al., manuscript in preparation), high long-term disease risk was associated with multiple T cell autoreactivities, while multiple autoantibodies appeared only closer to overt disease. Among these autoreactivities, nonanergic responses to proinsulin and IA2 were strongly associated with disease risk, while multiple anergic T cell autoreactivities were also found in subjects with moderate disease

risk. However, data presented here directly associated targeting of the ABBOS/Tep69 mimicry epitopes with disease risk. This could imply that such anergic cells actually serve as effector cells. If these cells enter into insulitis lesions, the chance to encounter relevant APCs as well as be rescued by nearby, nonanergic T cells specific for proinsulin or IA2 may be considerable and allow expression of effector function. The need for coincidence of cognate activation of both classes of T cells would add a stochastic element to islet destruction and perhaps relate to the long periods of time required for completion of β cell destruction and overt disease.

The previously reported anergy of diabetes-associated T cell pools (20, 46, 61) is unexpectedly broad. And unexpectedly, anergy is not a functional property that changes over the course of pre-diabetes and overt disease, but it rather appears to be a characteristic of some of the major autoantigens targeted in IDDM. Equally unexpected was the long persistence of autoimmune T cells up to 26 years after onset of overt disease, data consistent with the clinical experience of a rapid emergence of autoimmunity postislet transplantation. However, it remains difficult to envision the Ag sources that maintain, for example, proinsulin-reactive T cells, or for that matter, GAD65-specific T cells when even mature β cells express barely detectable levels. Collectively, the data presented here argue that validated assays of diabetes-associated T cell abnormalities offer important new insights and questions in diabetic autoimmunity.

Acknowledgments

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