Cellular Immune Responses to Human Islet Proteins in Antibody-Positive Type 2 Diabetic Patients

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Type 1 diabetes is a cell-mediated autoimmune disease characterized by autoantibody and peripheral blood mononuclear cell (PBMC) reactivity to islet cell proteins. Type 2 diabetes is not an autoimmune disease but rather results from both insulin resistance and a nonautoimmune insulin secretory defect. There is, however, a group of phenotypic type 2 diabetic patients who have islet autoantibodies that are similar to those of type 1 diabetic patients. In this study, we investigated, using cellular immunoblotting, whether type 2 diabetic patients positive for islet autoantibodies have PBMC responses to islet proteins. We observed that autoantibody negative (Ab^-) type 2 diabetic patients (n = 9) and normal control subjects (n = 12) demonstrated PBMCs responsive to 0–3 molecular weight regions. In contrast, autoantibody positive (Ab^+) type 2 diabetic patients (n = 11) demonstrated PBMC responses to 3–18 molecular weight regions, similar to that of type 1 diabetic patients (responsive to 4–18 molecular weight regions). PBMCs from over 90% of the Ab^+ type 2 and type 1 diabetic patients were observed to proliferate to islet proteins in the vicinity of 97 kDa. In contrast, 65–90% of type 1 diabetic patients had responsive PBMCs for islet proteins in most of the molecular weight regions, whereas <60% of the Ab^+ type 2 diabetic patients had PBMCs responsive to the same molecular weight proteins. Ab^+ type 2 diabetic patients appear to be heterogeneous with respect to cellular reactivity to islet proteins. Some subjects demonstrate PBMC responses similar to those of "classic" type 1 diabetic patients, whereas others have PBMC responses potentially distinct from type 1 diabetic patients. *Diabetes* 48:983–988, 1999

Diabetes is composed primarily of two clinically separate diseases: type 1 and type 2 diabetes (1). The diagnosis of type 1 versus type 2 diabetes is usually made phenotypically using criteria such as age at onset, apparent abruptness of onset of hyperglycemic symptoms, presence of ketosis, degree of obesity, and the need for insulin replacement. The pathogenesis of type 1 versus type 2 diabetes is believed to be different. Type 1 diabetes is a cell-mediated autoimmune disease directed against the β-cells and characterized by autoantibody and T-cell reactivity to islet proteins (2–5). In contrast, classic type 2 diabetes is not an autoimmune disease but rather results from both insulin resistance and a nonautoimmune insulin secretory defect (6).

However, there is a subgroup of phenotypic type 2 diabetic patients who have autoantibodies to islet cell proteins that are similar to those of type 1 diabetic patients (7–9). This group of patients comprises ~10–30% of Caucasian type 2 diabetic patients. The presence of islet autoantibodies in these type 2 diabetic patients suggests that they may have an autoimmune process responsible for their insulin secretory deficit (7,8). Previously, we demonstrated, using cellular immunoblotting, that peripheral blood mononuclear cells (PBMCs) from newly diagnosed type 1 diabetic patients are responsive to a wide spectrum of islet proteins (4). In this investigation, we asked whether type 2 diabetic patients positive for one or more islet autoantibodies have PBMC responses to multiple islet proteins that are similar to responses of type 1 diabetic patients. We compared, using cellular immunoblotting, the PBMC responses to islet proteins of type 1 diabetic patients, normal control subjects, and autoantibody negative (Ab^-) and autoantibody positive (Ab^+) type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

Subjects. These studies were approved by the University of Washington Human Subjects Review Committee. Normal control subjects were without chronic illness. All patients donated blood within 1 year of diagnosis of diabetes. Diagnosis of type 2 diabetes was based on the following criteria: age >35 years, absence of ketonuria or ketoacidosis, and not requiring insulin treatment at diagnosis. After diagnosis of type 2 diabetes, islet autoantibodies were determined. Type 2 diabetic patients were divided into two groups based on the presence of one or more islet cell autoantibodies (Ab^+ or Ab^-). Type 1 diabetic patients were classified according to American Diabetes Association criteria (1). The subject groups are as follows: control subjects (n = 12), type 1 diabetic patients (n = 12), Ab^+ type 2 diabetic patients (n = 11), and Ab^- type 2 diabetic patients (n = 9).

Islet cell autoantibody assay. Cryostat sections (4 μm thick) of human blood group O pancreas were adhered to dichromic acid–washed slides coated with poly-L-lysine hydrobromide. Test sera, preabsorbed with rat liver acetone powder (Sigma, St. Louis, MO) at dilutions of 1/4 and 1/16, were applied to slides, and the slides were incubated overnight in a covered moist chamber at 4°C. After rinsing with phosphate-buffered saline (PBS), the appropriate dilution of goat anti-human immunoglobulin G conjugated with fluorescein isothiocyanate was applied and incubated overnight at room temperature in a moist chamber. Slides were rinsed with PBS, and a coverslip was applied using 1,4-diazabicyclo-[2,2,2]octane (DABCO) mounting media (Sigma, St. Louis, MO). Slides were coded, randomly arranged, and read in a masked fashion using a Nikon Episcopic Fluorescent microscope. Each section was scored before breaking the code. Positive sera were end-point titered. Our laboratory has participated in the International Diabetes Workshops and the International Diabetes Society (IDS)-sponsored workshops and proficiency programs for islet cell autoantibody (ICA) with a sensitivity of ~63% and a specificity of 100%. The lower detection limit for our ICA assay is 1 Juvenile Diabetes Foundation (JDF) unit.

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Ab^-, autoantibody negative; Ab^+, autoantibody positive; BSA, bovine serum albumin; CPM, counts per minute; IA-2, islet cell autoantibody 512; ICA, islet cell autoantibody; IDS, International Diabetes Society; JDF, Juvenile Diabetes Foundation; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PHA, phytohemagglutin; SI, stimulation index.

GAD65 autoantibody assay. GAD65 autoantibodies were measured in a radiobinding immunoassay on coded serum samples (10). Briefly, ^{35}S-labeled GAD65 was produced in an in vitro coupled transcription and translation system with SP6 RNA polymerase– and nuclease-treated rabbit reticulocyte lyste (Promega, Madison, WI). In each analysis, triplicate samples of trichloroacetic acid precipitable ^{35}S-GAD65, 20,000 cpm, were diluted in 60 µl immunoprecipitation buffer (20 mmol/l Tris, 150 mmol/l NaCl, 0.15% [vol/vol] Tween-20, 0.1% [wt/vol] aprotinin, 10 mmol/l benzamidine, 0.1% [wt/vol] bovine serum albumin [BSA], pH 7.4) before the addition of 2.5 µl human serum (final serum dilution 1:25). Free ^{35}S-GAD65 was separated from the antibody-bound tracer by protein A Sepharose and several washes in 20 mmol/l Tris, 150 mmol/l NaCl, 0.1% (vol/vol) Tween-20, and 0.1% (wt/vol) BSA buffer (pH 7.4), using 96-well plates containing 0.65 µm hydrophilic polyvinylidene fluoride filters (Millipore, Bedford, MA). The immunoprecipitated radioactivity was counted after transferring the filters to glass scintillation vials using a Millipore Multiscreen Multiple Punch System.

The levels of GAD65 autoantibodies were expressed as a relative index (GAD65 index) using one positive serum (JDF world standard for ICA) and three negative standard sera from healthy subjects. The GAD index was calculated, and a positive was considered at ≥0.04, which is the 95th percentile based on 200 normal control subjects. In the IDS-sponsored GAD65 autoantibody serum exchange, our laboratory scored 100% for both sensitivity and specificity.

Islet cell autoantibody 512 assay. Autoantibodies to islet cell autoantibody 512 (IA-2) were measured under conditions identical to those described for GAD65 autoantibody (10). The plasmid containing the cDNA coding for the cytoplasmic portion of IA-2 was donated by Dr. G. Eisenbarth (Barbara Davis Research Center, Denver, CO). The IA-2 autoantibody index for each sample was calculated using the same JDF standard serum and control sera that were used in the GAD65 autoantibody assay. An IA-2 index ≥0.01, the 95th percentile based on 200 normal control subjects, was taken as the cutoff for positivity.

Cellular immunoblotting. PBMC stimulation was investigated using cellular immunoblotting as described for islet proteins (4,11) and nonislet proteins (12–15). Briefly, human islet proteins are separated using SDS-PAGE (16) and gel-electroblotted onto nitrocellulose. Nitrocellulose particles are prepared (17) and then used to stimulate PBMCs in vitro. PBMCs from masked blood samples were isolated by density gradient centrifugation using Lymphoprep. Unfractionated PBMCs (3.0×10^5 cells/well) were placed in 96-well tissue culture plates (Costar, Cambridge, MA). To each tissue culture well, 100 µl of nitrocellulose particles from an individual blot section containing islet cell proteins or tetanus toxoid were added. One antigen preparation was used throughout the study. Cultures were prepared in triplicate and incubated for 6 days at 37°C and 5% CO_2. Phytohemagglutin (PHA) or concanavalin A were added to control wells on day 4 of culture. After 6 days, [^{3}H]thymidine (1 µCi per well) was added, and the cultures were incubated for 8 h. Cultures were harvested using a Tomtec 96-well cell harvester and counted using a Betaplate (Pharmacia, Uppsala, Sweden) liquid scintillation counter. A stimulation index (SI) for each molecular weight section was calculated as follows: SI = mean counts per minute (CPM) experimental wells / mean CPM control wells. Control wells contained nitrocellulose particles without antigen. Positive proliferation was considered to be an SI >2.0, which corresponds to greater than the mean + 3 SDs of control responses. As previously reported (4), the intra-subject coefficient of variation for this methodology is ~30%. Early studies to determine the optimal antigen dose and studies describing the specificity of the PBMC response of type 1 diabetic patients to islet proteins and to known islet autoantigens using cellular immunoblotting have been previously reported (4).

Chemicals. Electrophoresis chemicals and nitrocellulose were purchased from Bio-Rad (Richmond, CA). RPMI 1640, PHA, silver stain, DMSO, 2-β-mercaptoethanol, and normal human sera were purchased from Sigma. Penicillin/streptomycin sulfate, HEPES (*N*-2-dydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), D-glucose, L-glutamine, and sodium bicarbonate and carbonate were purchased from Gibco (Grand Island, NY). Ficoll and Lymphoprep were purchased from Accurate Chemical (Westbury, NY).

Statistics. The Mann-Whitney U and χ^2 tests were used for statistical analysis. Significance was considered $P < 0.05$. Variances reported in the text are SDs.

RESULTS

Characteristics of the Ab+ type 2 diabetic patients (n = 11) are shown in Table 1. Two subjects were positive for one autoantibody, seven subjects were positive for two autoantibodies, and two subjects were positive for three autoantibodies (Table 1). The Ab– type 2 diabetic patients had a mean age of 49.4 ± 9.0 years, and the Ab+ type 2 diabetic patients had a mean age of 49.9 ± 9.3 years. There were six women and three men in the Ab– subject group and seven women and four men in the Ab+ subject group. The mean BMI for the Ab– subjects was 35.7 ± 6.6, and the mean BMI of the Ab+ subjects was 25.9 ± 5.3. Characteristics of the type 1 diabetic patients (n = 12) are shown in Table 2. The mean age of the type 1 diabetic patients was 21.8 ± 8.5 years.

Using cellular immunoblotting, Ab– type 2 diabetic patients (n = 9) demonstrated PBMC responses to the separated islet proteins that were similar to those of control subjects (n = 12) (Fig. 1). In contrast, type 1 diabetic patients (n = 12) demonstrated PBMC responses to multiple islet proteins (Fig. 2*A*) that were consistent with our previous findings in type 1 diabetic patients (5). Ab+ type 2 diabetic patients (n = 11) also demonstrated PBMC responses to multiple islet proteins (Fig. 2*B*). The overall magnitude of the responses to the 18-blot sections was significantly less in the Ab+ type 2 diabetic patients (mean SI = 2.1 ± 0.3) compared with that of the type 1 diabetic patients (mean SI = 3.4 ± 0.4). Evaluation of the number of molecular weight regions stimulatory to PBMCs from each subject is depicted in Fig. 3. Ab– type 2 diabetic patients responded to 0–3 molecular weight regions with a mean of 0.3 ± 0.7 regions. Normal control subjects also responded to 0–3 molecular weight regions with a mean of 1.1 ± 1.0 regions. In contrast, type 1 diabetic patients responded

TABLE 1
Characteristics of the Ab+ type 2 diabetic patients

Subject	Sex	Disease duration (months)	Age (years)	ICA (JDF)	GAD65 (index)	IA-2 (index)
1	M	5	64	6	− (0.02)	− (−0.032)
2	F	2	49	—	+ (0.05)	− (0.005)
3	F	2	50	—	+ (0.15)	+ (0.013)
4	M	9	51	10	+ (2.12)	− (−0.01)
5	F	7	48	10	+ (0.46)	− (−0.077)
6	M	10	35	10	+ (0.40)	− (−0.063)
7	M	11	52	20	+ (0.95)	− (−0.063)
8	F	4	50	20	+ (1.23)	− (0.005)
9	F	5	48	20	+ (1.36)	− (−0.01)
10	F	8	67	6	+ (1.05)	+ (0.015)
11	F	9	35	10	+ (1.03)	+ (0.711)
		6.5 ± 3.0	50 ± 8.9	12.4 ± 5.6	0.8 ± 0.62	0.044 ± 0.21

Data are means ± SD. Disease duration is defined as the time from onset of clinical diabetes to blood drawn for study.

TABLE 2
Characteristics of the type 1 diabetic patients

Subject	Sex	Disease duration (months)	Age (years)	ICA	GAD65 (index)	IA-2 (index)
1	F	3	8	+	− (0.01)	+ (0.01)
2	F	5	18	+	− (0.02)	+ (0.52)
3	F	10	21	+	− (0.01)	− (−0.01)
4	F	3	24	+	− (0.00)	+ (0.12)
5	F	2	28	+	− (−0.05)	+ (0.26)
6	F	5	34	+	− (−0.01)	− (−0.02)
7	F	1	33	+	− (−0.015)	− (−0.02)
8	M	10	20	+	− (−0.05)	− (−0.01)
9	M	11	20	+	+ (0.28)	− (−0.027)
10	M	4	33	+	+ (0.49)	+ (0.21)
11	M	3	12	+	+ (0.50)	+ (0.58)
12	M	11	11	−	+ (0.54)	+ (0.15)
		5.7 ± 3.6	21.8 ± 8.5		0.14 ± 0.23	0.15 ± 0.2

Data are means ± SD. Disease duration is defined as time from onset of clinical diabetes to blood drawn for study.

to 4–18 molecular weight regions (mean 12.4 ± 4.8), whereas Ab^+ type 2 diabetic patients responded to 3–18 molecular weight regions (mean 10.1 ± 4.4). The number of molecular weight regions recognized by both the type 1 diabetic patients and the Ab^+ type 2 diabetic patients was significantly higher than the number of molecular weight regions recognized by the control subjects or Ab^- type 2 diabetic patients. The proliferative responses to tetanus of PBMCs from subjects in all four groups were not different: mean SI of control subjects was 9.0 ± 7.3, of Ab^- type 2 diabetic patients was 5.9 ± 2.6, of Ab^+ type 2 diabetic patients was 3.2 ± 1.4, and of type 1 diabetic patients was 6.9 ± 5.9.

Figure 4 depicts the percentage of type 1 and Ab^+ type 2 diabetic patients demonstrating positive PBMC responses to proteins in each blot section. Over 90% of the type 1 and Ab^+ type 2 diabetic patients had PBMCs that recognized islet proteins in the vicinity of the 97 kDa molecular weight region. The percentage of Ab^+ type 2 diabetic patients with PBMCs responsive to islet proteins in many of the molecular weight regions was significantly less than the percentage of type 1 diabetic patients who were responsive to the same molecular weight regions.

DISCUSSION

Ab^+ type 2 diabetic patients have been hypothesized to have a slowly progressive form of type 1 diabetes (8,18,19). These Ab^+ type 2 diabetic patients tend to have higher HbA_{1c} levels (20), lower C-peptide (21), and have a propensity toward insulin dependency (9,21–23) compared with Ab^- "classical" type 2 diabetic patients. Zavala et al. (22) demonstrated in a group of Ab^+

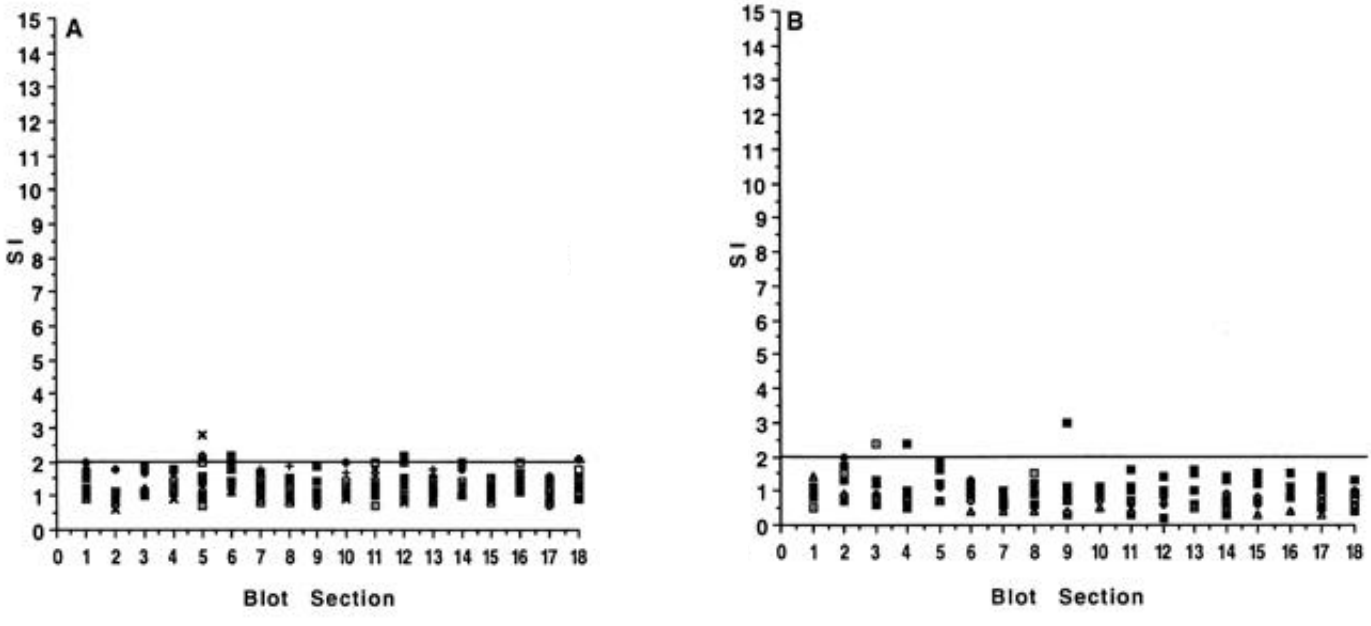

FIG. 1. PBMC responses of 12 control subjects (*A*) and 9 Ab^- type 2 diabetic patients (*B*) to separated islet proteins using cellular immunoblotting. Blot sections correspond to molecular mass regions >200 kDa (1) and <14 kDa (18). Responses are shown in SI units. Individual responses are depicted as separate symbols, and each data point represents the mean of triplicate wells. The line represents an SI of 2.0.

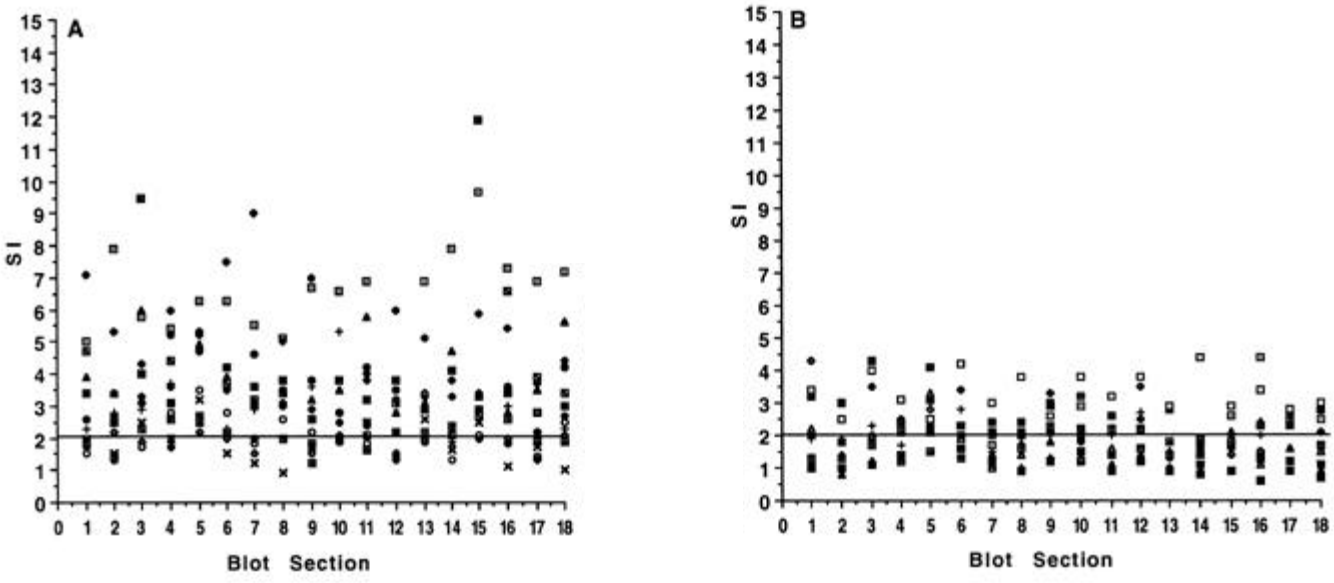

FIG. 2. PBMC responses of 12 type 1 diabetic patients (*A*) and 11 Ab+ type 2 diabetic patients (*B*) to separated islet proteins using cellular immunoblotting. Blot sections correspond to molecular mass regions >200 kDa (1) and <14 kDa (18). Responses are shown in SI units. Individual responses are depicted as separate symbols, and each data point represents the mean of triplicate wells. The line represents an SI of 2.0.

type 2 diabetic patients, whose disease could not be controlled with oral hypoglycemic agents, that PBMCs from these subjects inhibited stimulated insulin secretion by rat islet cells, suggesting a cell-mediated autoimmune component.

Previously, we have demonstrated, using cellular immunoblotting, that type 1 diabetic patients have PBMCs that recognize a wide spectrum of islet proteins (4). Therefore, we questioned whether Ab+ type 2 diabetic patients would demonstrate PBMC reactivity to islet proteins in a manner similar to PBMC responses of type 1 diabetic patients. Using cellular immunoblotting, we observed that PBMCs from Ab− type 2 diabetic patients and control subjects proliferate to a very limited number of separated islet proteins. In contrast, Ab+ type 2 diabetic patients appeared to have PBMC reactivity to numerous

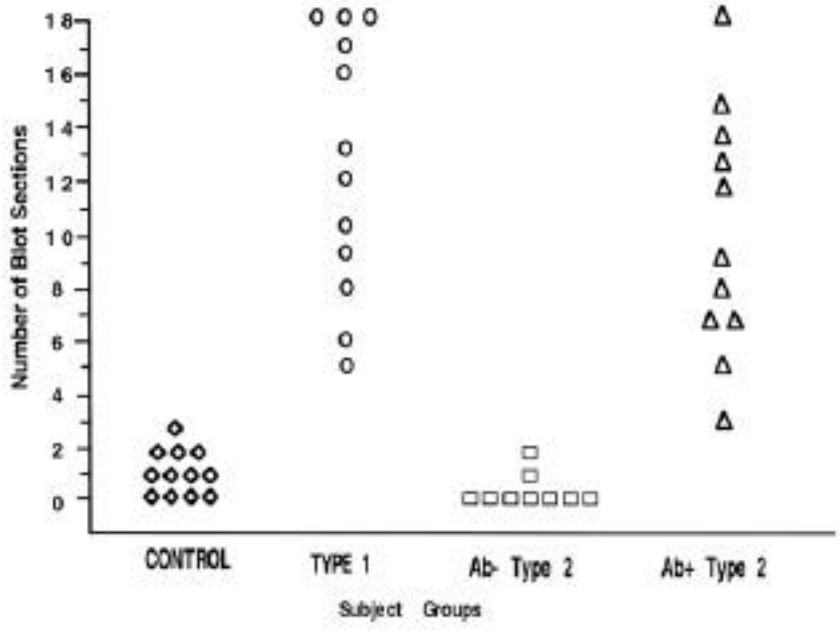

FIG. 3. PBMC responses of 12 normal control subjects, 12 type 1 diabetic patients, 9 Ab− type 2 diabetic patients, and 11 Ab+ type 2 diabetic patients. The number of molecular weight regions positive for each individual are shown. The different symbols represent individual subjects. A positive response is taken as SI >2.0.

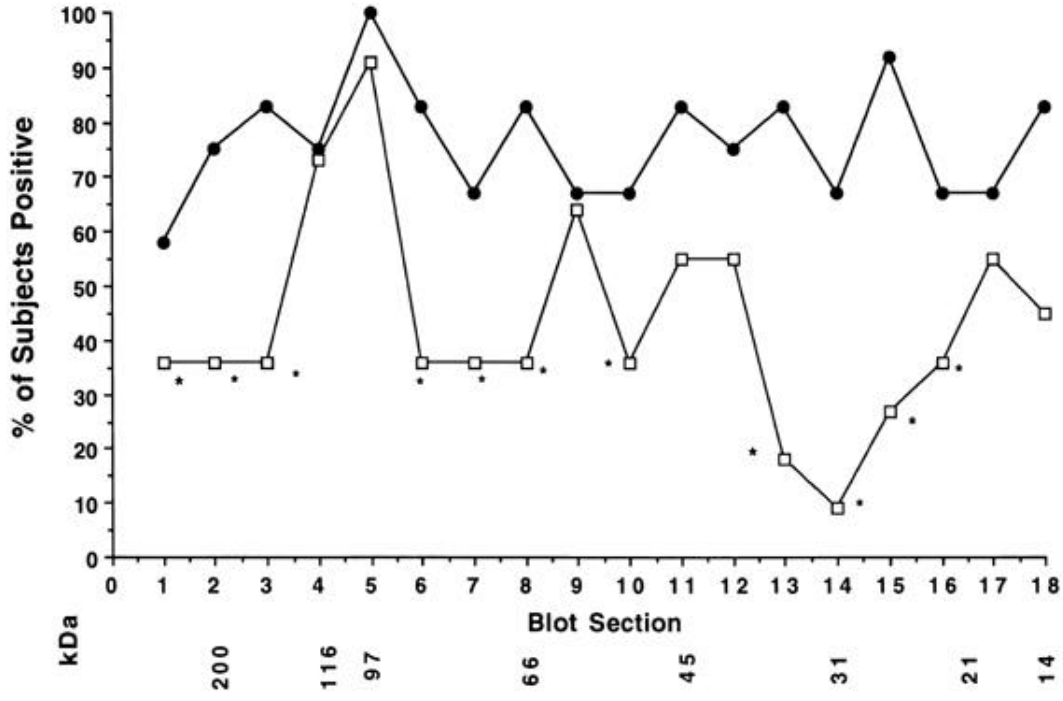

FIG. 4. PBMC responses of 12 type 1 diabetic patients (●) and 11 Ab$^+$ type 2 diabetic patients (□). The percentage of subjects responding to each molecular weight region is shown. A positive response is taken as SI >2.0. Blot sections correspond to molecular mass regions >200 kDa (1) and <14 kDa (18). *P < 0.05 significant differences.

islet proteins, similar to that of type 1 diabetic patients. This response of Ab$^+$ type 2 and type 1 diabetic patients to numerous islet proteins is not likely due to a nonspecific exaggerated T-cell response, since their response to tetanus was similar to that of control subjects and Ab$^-$ type 2 diabetic patients.

In this study, we compared autoantibody and T-cell responses to separated islet proteins from type 1 diabetic patients, control subjects, Ab$^-$ type 2 diabetic patients, and Ab$^+$ type 2 diabetic patients. Comparison of the autoantibody results of the type 1 and Ab$^+$ type 2 diabetic patients demonstrated higher indices for GAD autoantibodies in Ab$^+$ type 2 versus type 1 diabetic patients. The opposite appeared to be true when comparing the IA-2 antibody indices between the two groups (Tables 1 and 2). In a much larger study of antibodies in type 2 diabetic patients, we have observed that IA-2 antibodies are far less common than ICAs or GAD antibodies (9). For the PBMC responses between the Ab$^+$ type 2 and type 1 diabetic patients, we observed that the overall magnitude of the PMBC responses to the islet proteins was lower in the Ab$^+$ type 2 compared with the type 1 diabetic patients. We have observed decreases in cellular reactivity to islet proteins in type 1 diabetic patients as the time from diagnosis of type 1 diabetes increases (B.B.-W., J.P.P., unpublished observations). However, in this study, we found no difference in time span from clinical diagnosis of diabetes to time of blood drawn between the type 1 diabetic patients (mean 5.7 ± 3.6 months) and the Ab$^+$ type 2 diabetic patients (mean 6.5 ± 3.0 months). But, the time of clinical diagnosis of type 2 diabetes is recognized as being less certain than that for type 1 diabetes. We also observed that the PBMC responses to the islet proteins appeared to be more heterogenous in the Ab$^+$ type 2 versus type 1 diabetic patients. The heterogeneity observed in the cellular reactivity between type 1 and Ab$^+$ type 2 diabetic patients may reflect differences in the pathogenic disease processes between the two groups.

PBMCs from the Ab$^+$ type 2 and type 1 diabetic patients demonstrated a high percentage of cells responding to the proteins in the vicinity of 97 kDa, suggesting that this region may contain islet proteins important for both diabetes disease processes. However, significant differences in PBMC responses of the Ab$^+$ type 2 and type 1 diabetic patients to proteins in many other molecular weight regions were also observed. These findings raise the possibility of pathogenic or antigenic differences between type 1 and some Ab$^+$ type 2 diabetic patients. More subjects need to be studied to investigate these differences.

In conclusion, some phenotypic type 2 diabetic patients have both humoral and cellular autoreactivity to islet proteins, suggesting that the pathophysiology between type 1 and Ab$^+$ type 2 diabetic patients may be similar. However, the heterogeneous nature of the PBMC recognition of specific proteins and the magnitude of the cellular responses may indicate that some of the Ab$^+$ type 2 diabetic patients have an autoimmune process distinct from "classic" type 1 diabetes.

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