Original Article

Analysis of T-Cell Assays to Measure Autoimmune Responses in Subjects With Type 1 Diabetes

Results of a Blinded Controlled Study

Vicki Seyfert-Margolis,[1] Trang D. Gisler,[1] Adam L. Asare,[1] Richard S. Wang,[1] H. Michael Dosch,[2] Barbara Brooks-Worrell,[3] George S. Eisenbarth,[4] Jerry P. Palmer,[3] Carla J. Greenbaum,[5] Stephen E. Gitelman,[6] Gerald T. Nepom,[5] Jeffrey A. Bluestone,[6] and Kevan C. Herold[7]

Type 1 diabetes is a chronic autoimmune disease mediated by autoreactive T-cells. Several experimental therapies targeting T-cells are in clinical trials. To understand how these therapies affect T-cell responses in vivo, assays that directly measure human T-cell function are needed. In a blinded, multicenter, case-controlled study conducted by the Immune Tolerance Network, we tested responses in an immunoblot and T-cell proliferative assay to distinguish type 1 diabetic patients from healthy control subjects. Peripheral blood cells from 39 healthy control subjects selected for DR4 and 23 subjects with recently diagnosed type 1 diabetes were studied. Autoantibody responses were measured in serum samples. Positive responses in both assays were more common in peripheral blood mononuclear cells from new-onset type 1 diabetic patients compared with control subjects. The proliferative, immunoblot, and autoantibody assays had sensitivities of 58, 91, and 78% with specificities of 94, 83, and 85%, respectively. When cellular assays were combined with autoantibody measurements, the sensitivity of the measurements was 75% with 100% specificity. We conclude that cellular assays performed on peripheral blood have a high degree of accuracy in discriminating responses in subjects with type 1 diabetes from healthy control subjects. They may be useful for assessment of cellular autoimmune responses involved in type 1 diabetes. *Diabetes* 55:2588–2594, 2006

From the [1]Immune Tolerance Network, Bethesda, Maryland; the [2]Hospital for Sick Children, University of Toronto, Toronto, Canada; the [3]University of Washington, Seattle, Washington; the [4]Barbara Davis Research Center, University of Colorado, Denver, Colorado; the [5]Benaroya Research Center, Seattle, Washington; the [6]Diabetes Center, University of California at San Francisco, San Francisco, California; and the [7]Naomi Berrie Diabetes Center, Columbia University, New York, New York.

Address correspondence and reprint requests to Kevan C. Herold, MD, College of Physicians and Surgeons, Columbia University, PH8 Rm 864, 630 W. 168th St., New York, NY 10032. E-mail: kh318@columbia.edu.

Received for publication 24 October 2005 and accepted in revised form 25 May 2006.

DASP, Diabetes Autoantibody Standardization Program; ELISpot, enzyme-linked immunospot; IA2, insulinoma-associated protein 2; ICA, islet cell antibody; IL, interleukin; ITN, Immune Tolerance Network; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; ROC, receiver operating characteristic.

DOI: 10.2337/db05-1378

Type 1 diabetes is a chronic autoimmune disease in which insulin-producing islet β-cells of the pancreas are destroyed (1,2). There is no known treatment that can prevent this autoimmune process; thus, the disease results in a lifelong dependence on exogenous insulin. Current diagnosis relies on measures of hyperglycemia, which results when significant immune-mediated destruction of β-cells has occurred. Despite the understanding that the pathological process involves T-cells that cause destruction of β-cells, we do not have a way to identify cells that may be involved in this process or that are even reactive to the identified autoantigens (3). Instead, we rely primarily on metabolic measurements to characterize the extent of β-cell destruction or autoantibodies. To date, immune markers of type 1 diabetes have primarily centered on the presence of islet cell antibodies (ICAs) or antibodies against proteins found in β-cells: GAD, tyrosine phosphatase-like molecule ICA512/insulinoma-associated protein 2 (IA2), and insulin (4–7). Measurement of these autoantibodies has been shown to be useful for prediction of diabetes, but once the disease is present, their utility in assessing the progression of disease is not clear, and immune interventions that have affected the disease have not caused changes in autoantibodies (8,9). A measure of the relevant cellular immune responses would be useful for gaining a better understanding of the disease process and the effects of immune therapies, and it might suggest drug targets that could interrupt the disease before critical β-cell destruction.

In animal models of type 1 diabetes, T-cells have been shown to be the primary mediators of disease, but measurement of T-cells specific for type 1 diabetes autoantigens in humans with type 1 diabetes has proven difficult (10). Measurement of autoreactive human T-cell responses requires extensive in vitro culture systems and includes readouts of cytokine production or proliferative responses (10–14).

In an international workshop for standardization of T-cell assays for type 1 diabetes, cellular responses to GAD65, IA2, insulin, p69, and tetanus toxoid were studied (10). The workshop results highlighted difficulties in preparing suitable antigens for study—even T-cell clones specific for GAD65 were not able to respond to the different preparations of the antigen. Of most concern is the observation that peripheral T-cell reactivity to the

autoantigens was not different in patients with type 1 diabetes compared with healthy control subjects. The disappointing results in the workshop setting compared with reports in the literature may have been related to the use of fresh versus frozen cells, the preparations of the antigens that were used, and/or the absence of objectivity in the previous studies because of knowledge of the source of the blood sample that was studied.

As the Immune Tolerance Network (ITN) has embarked on a number of phase I and phase II immunotherapy trials in type 1 diabetes (www.immunetolerance.org), the need for validated cellular assays of disease activity have become increasingly important. Therefore, we directly assessed four cellular assays in a blinded workshop, including major histocompatibility complex (MHC)-GAD peptide tetramer analysis (15), GAD-specific enzyme-linked immunospot (ELISpot) analysis (16), a cellular immunoblot assay (17,18), and a T-cell proliferation assay (14) to identify different immune responses in patients with diabetes compared with normal control subjects. To minimize variables that may have led to poor performances in previous studies, antigen preparations were made by the individual investigators, and to eliminate bias, the samples were collected at multiple sites and sent to the laboratories in a blinded manner. Type 1 diabetes–associated biochemical autoantibodies were also measured in the type 1 diabetic patients and control subjects. The abilities of these assays to measure responses in patients with diabetes that were not seen in healthy subjects were studied, and the accuracy of classification for each assay alone or in combination was determined. Unfortunately, for technical reasons involved in the use of frozen cells, the tetramer and ELISpot assays were uninformative; thus, this article describes the results of the other two T-cell assays and the autoantibody analyses.

RESEARCH DESIGN AND METHODS

A total of 23 participants with new-onset type 1 diabetes and 39 healthy control participants were enrolled into a blinded case-control study with blood collection at a single time point. The entry criteria included age 8–30 years with a diagnosis of type 1 diabetes according to American Diabetes Association criteria for at least 1 week and <6 months. Participants had weight >25 kg and detectable anti-GAD, anti-ICA512/1A2, or insulin antibodies before insulin treatment. The insulin antibodies (microinsulin autoantibodies) were not used for calculations because all subjects with type 1 diabetes were insulin treated at the time of study. The healthy control subjects included individuals without a personal or family history of type 1 diabetes or other autoimmune diseases. Because of restrictions in obtaining blood samples from healthy individuals, the age of the normal control subjects was higher than subjects with type 1 diabetes. The demographic information on the study subjects is shown in Table 1. Both groups of participants were HLA typed, and the healthy control group was selected to achieve a similar rate of DR4 positivity as the patients with type 1 diabetes. All participants gave informed consent, and the protocol was approved by the institutional review boards at each participating institution.

Blood collection was performed on a single occasion. Subjects were enrolled at three institutions, and the analysis laboratories were notified in advance of the planned collection of blood, but they remained blinded to the subjects' identity. Samples from two to four participants were tested concurrently. Peripheral blood was collected in sodium-heparin tubes and shipped overnight at ambient temperature to the laboratories in styrofoam containers. Serum was isolated at the sites, frozen, and shipped to the autoantibody laboratory without identification of the subject.

Peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation from sodium-heparinized venous blood samples in each laboratory.

Cellular immunoblotting assay. Cellular immunoblotting was performed as previously described (17). Human pancreata were obtained by University of Washington transplant surgeons, and pancreatic islets were isolated within 48 h postmortem at the Islet Satellite of the Tissue Culture Core of the DERC (Diabetes Endocrinology Research Center), University of Washington, Seattle, Washington. Islet cells and tetanus toxoid were subjected to preparative 10% SDS-PAGE (19). After electrophoresis, the gels were electroblotted onto nitrocellulose (BioRad, Richmond, CA) at 30 mA overnight. The nitrocellulose membranes were cut into strips based on molecular weight and then solubilized, using carbonate/bicarbonate buffers. The resulting nitrocellulose particles were then used to stimulate PBMCs in vitro (20).

TABLE 1
Patient demographics and HLA haplotype

	Patients with type 1 diabetes	Healthy normal control subjects
n	23	39
Age*	18.7 ± 9.7	21.5 ± 3.9
Female*	39 (9)	67 (26)
HLA type*		
DR4 and DR3	22 (5)	3 (1)
DR4 only	39 (9)	38 (15)
DR3 only	30 (7)	26 (10)
Neither DR4 nor DR3	9 (2)	33 (13)
Race		
White	91 (21)	77 (30)
Hispanic	9 (2)	13 (5)
Asian	0	10 (4)
Relatives with type 1 diabetes†		
Yes	13 (3)	0
No	70 (16)	100 (39)
Unknown	17 (4)	0

Data are means ± SE or % (*n*). *$P = 0.025$; †$P < 0.01$ points with type 1 diabetes vs. control subjects.

PBMCs were diluted and placed into a 96-well flat-bottom tissue culture plate at a concentration of 3.5×10^5 per well. Nitrocellulose particles containing islet cell proteins from an individual blot section, wells containing nitrocellulose particles without antigen, or cells alone were included in the assay. The cultures were prepared in triplicate and incubated for 5 days at 37°C and 5% CO_2. The positive control antigens used were tetanus toxoid, which was added at the initiation of the cultures, and phytohemagglutinin, which was added on day 3 of culture. After 5 days, tritiated-thymidine (1 μCi per well) was added to all of the wells, and the cultures were harvested 18 h later. The counts per minute were determined using a Betaplate (LKB Pharmacia) liquid scintillation counter. A stimulation index for each molecular weight section was calculated as follows: SI = (mean CPM in experimental wells)/(mean CPM in control wells), where SI is the stimulation index, CPM is the counts per minute, and control wells are wells without antigen.

Positive proliferation is considered to be a stimulation index >2.0, which corresponds to the mean + 3 SDs of control values (17). The subject was considered to have a positive response in the assay when more than three blot sections had a stimulation index >2.0 (17). In previous studies, PBMCs from normal control subjects have shown a stimulation index >2.0 in up to three islet blot sections, whereas PBMCs from patients with type 1 diabetes have generally shown stimulation indexes >2 in 4–18 blot sections. PBMC response to tetanus toxoid was used as an antigen-specific positive control, and PBMC response to mitogens was included to test for viability of the cultures. The counts per minute (average ± SE) in the control wells without antigen (i.e., nitrocellulose only) in subjects with type 1 diabetes and control subjects were 1,227 ± 594 and 1,356 ± 812 cpm, and the stimulation index (average ± SE) in response to tetanus toxoid and mitogen in subjects with type 1 diabetes and control subjects was 24 ± 19 and 58 ± 45 and 19 ± 21 and 41 ± 37, respectively (P = NS).

T-cell proliferation assay. Mononuclear cells were enriched on Ficoll-Paque gradients and seeded at a concentration of 1×10^5 per well of a culture-grade, flat-bottom, 96-well culture plate in Hybrimax 2897 protein-free media (Sigma, St. Louis, MO). To allow detection of preactivated as well as anergic T-cells, cultures received 10 units/ml recombinant human interleukin (IL)-2 with dilutions made in Hybrimax medium (21–23). The responses measured in the presence of IL-2 supplements retain an absolute and dose-dependent requirement for antigen (14). Plates were incubated in standard CO_2 incubators for 1 week, adding 1 μCi [^{3}H]thymidine for the last 12 h before automated harvesting and scintillation counting. PBMC cultures were stimulated with 15

TABLE 2
Sensitivity and specificity of the assays

	n	Sensitivity	Composite 95% CI	Specificity	Composite 95% CI
T-cell proliferation	52	0.58	0.37–0.79	0.91	0.79–1.0
Immunoblot	47	0.94	0.83–1.0	0.83	0.69–0.97
GAD65	62	0.74	0.57–0.91	0.85	0.72–0.95
ICA512	62	0.52	0.30–0.74	0.97	0.92–1.0

test antigens, one per replicate set, using 0.01–20 μg per well. Antigens/peptides were added to dry plates in 50-μl volumes and stored frozen until use (21,23). Test antigens/peptides included proinsulin, insulin B p9-23, GAD65, GAD65 p555-567, ICA69 p349-361 (Tep69), and IA2 (islet antigens); casein, β-lactoglobulin, BSA, BSA p147-165 (ABBOS), and BSA p193-205 (dietary antigens); glial fibrillary acidic protein and S100β (glial antigens); and myelin basic protein and myelin basic protein–exon 2 (central nervous system antigens) (21,23,24). Phytohemagglutinin and tetanus toxoid provided positive, actin, hemoglobin, and cytochrome-c–negative control antigens. To compare proliferative responses in different samples, data were normalized as stimulation indexes (cpm antigen-stimulated/cpm unstimulated). A positive response was defined to be >1.5 and greater than the mean stimulation index in ovalbumin-stimulated culture replicates +3 SDs. The background counts, ranging from 500 to 1,200 cpm, were not significantly different from those in ovalbumin-stimulated cultures ($P > 0.3$). For each sample, positive responses were added, deriving a T-cell response score (range 0–15); a score of ≥4 was set to be a positive response.

GAD65 autoantibody and ICA512 autoantibody assay. GAD65 and IA2AA autoantibodies were measured using methods previously described (5). The cut points for positivity were set at indexes of 0.032 (GAD65 autoantibody) and 0.049 (ICA512 autoantibody), the 99th percentile from receiver operating characteristic (ROC) curves in 198 healthy control subjects and 50 patients with new-onset diabetes (5). The presence of autoantibodies for GAD65, ICA512bdc (IA2), and insulin was determined by fluid-phase radioassay. In previous Diabetes Autoantibody Standardization Program (DASP) workshops (2000, 2002, 2003, and 2005), with blinded analysis of 100 control subjects and 50 patients with new-onset type 1 diabetes, the sensitivity and specificity for anti-GAD65 were 84, 90, 84, and 76, and 96, 93, 98, and 99%, respectively, and the sensitivity and specificity for anti-ICA512 were 52, 62, 58, and 64, and 100, 99, 100, and 100%, respectively.

Genotyping. DR typing was performed at the ITN HLA Typing Core facility. DNA isolation was performed using the QIAmp Blood Mini Kit (Quiagen). Complete class II haplotyping was performed. Additionally, the ITN HLA Typing Core facility used the sequence-based typing method to identify alleles in the highly polymorphic genes of the MHC. The reaction was run using a kit available from PE Biosystems on an ABI Prism 377 DNA sequencer.

Other assays. The study design included analysis of T-cell responses by ELISpot and class II MHC tetramer analysis. However, because of technical problems including those associated with freezing and thawing of cells, the assay results were not usable, and the data from these studies are not included.

Statistical analyses. Following Pepe (2003), composite 95% CIs for classification probabilities were calculated, using exact binomial methods. Assays were performed independently and masked as to disease status, allowing direct comparison of classification probabilities and areas under ROC curves for the two cellular assays. Because of poor quality of samples when they arrived in the laboratory, two were excluded from the T-cell proliferation assay and five were excluded from the immunoblot assay. In addition, samples were unavailable for the T-cell proliferation assay from 5 of 62 subjects, and from another 10 of 62 subjects for the immunoblot assay. In four samples, the results of the proliferation assay were indeterminate. Because all of the type 1 diabetic subjects were treated with insulin, the comparative analysis of autoantibodies was performed with only the anti-GAD and anti-ICA512/IA2 assays. χ^2 test statistics were calculated to assess differences in frequency of events in each group, and Fisher's exact test was used for 2 × 2 tables. R statistical software (available from http://www.r-project.org/) was used for all analyses and summary statistics. $P < 0.05$ was considered to be of statistical significance.

RESULTS

Patient demographics. Samples from 62 subjects (23 subjects with type 1 diabetes and 39 healthy control subjects) were collected and used for this study (Table 1). The age of the healthy control subjects was slightly greater than the type 1 diabetic subjects because of restrictions on sampling in normal children, but the difference was not statistically significant ($P = 0.19$). In addition, there was a significantly greater number of male subjects in the patients with type 1 diabetes compared with the healthy control subjects ($P < 0.05$). The control subjects were selected for expression of one diabetes-associated HLA allele (DR4), but an analysis of the cohorts showed that there was a significant increase in the proportion of subjects with type 1 diabetes who were HLADR3$^+$ or HLADR3$^+$ and HLADR4$^+$ ($P = 0.025$). Overall, 91% of the type 1 diabetic participants had diabetes-associated HLA (DR4 and/or DR3), and 9% were negative for both DR4 and DR3 (Table 1). Of 39 healthy control subjects, 67% were DR4$^+$ and/or DR3$^+$ and 33% were neither DR4$^+$ nor DR3$^+$ (Table 1).

Immunoblot results. The immunoblot assay was performed in 18 subjects with type 1 diabetes and 29 healthy control subjects. Of the healthy control participants, 24 of 29 (83%) tested negative, whereas 17 of 18 (94%) of the type 1 diabetic participants were scored as positive ($P < 0.0001$) (Fig. 1). The ROC of this assay is shown in Fig. 2. The thresholds for this assay appear optimal for classification of a positive response in subjects with type 1 diabetes. Overall, the sensitivity and specificity of this assay was 94 and 83%, respectively (Table 2).

Because of the difference in frequency of class II HLA genotypes in the patients with type 1 diabetes compared with healthy control subjects, it was possible that differences in alloresponsiveness to MHC antigens expressed by islets and transferred to the blots accounted for the differences in the proliferative responses that were measured rather than a disease-specific difference. Therefore, we compared the responses in the healthy control participants and the subjects with type 1 diabetes who were DR3$^+$ and/or DR4$^+$. When the analysis was limited to these individuals, there was still a significant difference in the rates of positive responses in subjects with type 1 diabetes (15 of 16 patients with type 1 diabetes showed a positive response vs. 5 of 20 control subjects, $P < 0.001$). However, among the healthy control participants who were misclassified, all five were DR3$^+$ or DR4$^+$.

T-cell proliferation assay. Of 57 participants tested for the T-cell proliferation assay, 30 of 33 (91%) healthy control participants tested negative, whereas 11 of 19 (58%) type 1 diabetic participants were scored as positive; 4 participants (2 type 1 diabetic and 2 healthy control subjects) were classified as indeterminate (T-cell score = 3), and 1 participant's sample yielded insufficient cell count for accurate classification ($P = 0.0002$) (Fig. 1). The ROC of this assay is shown in Fig. 2 and confirms that the selection of thresholds for a positive response are optimal for identifying responses in type 1 diabetic patients. The sensitivity and specificity of this assay were 58 and 91%, respectively (Table 2). There were three false-positive results with this assay. Two of these subjects were HLA

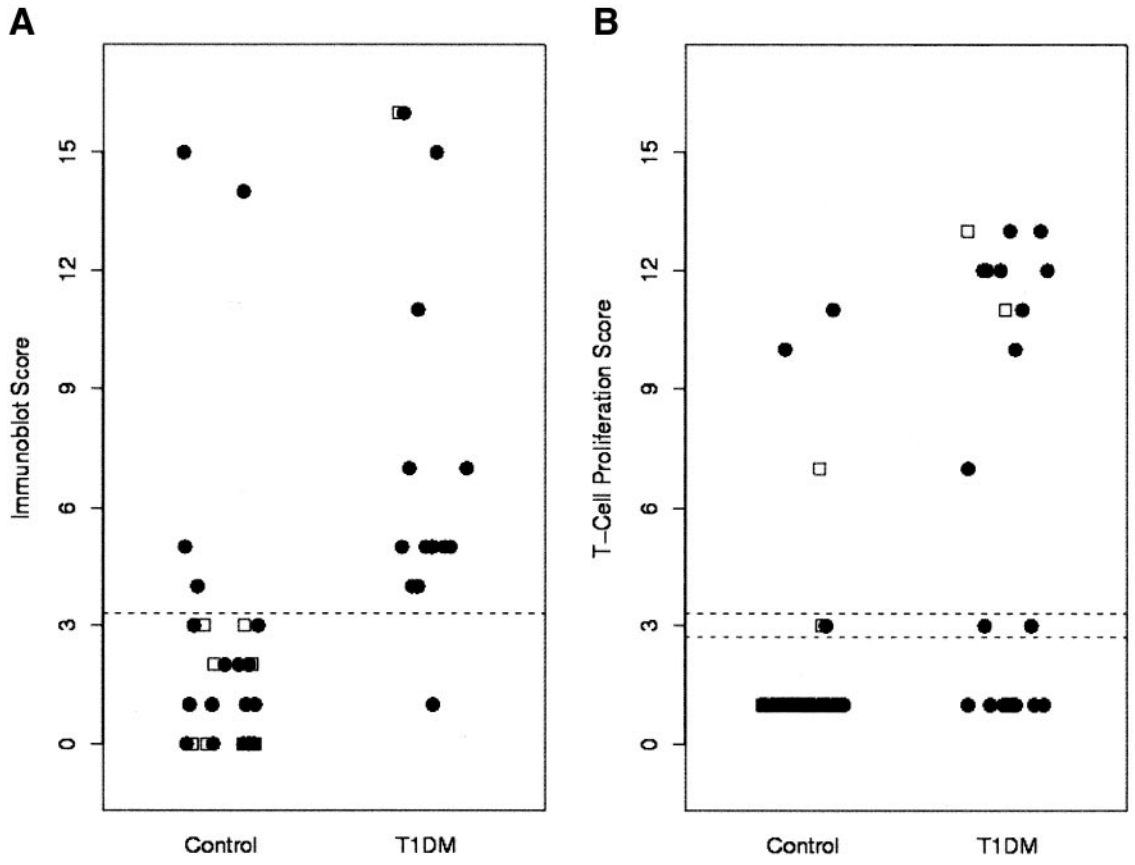

FIG. 1. Results from the immunoblot (*A*) and T-cell proliferative (*B*) assays. These assays were carried out with samples from normal control subjects and patients with type 1 diabetes. Values above the dotted lines were scored as positive. Values within the two dotted lines were designated as indeterminate and were not included in the statistical analysis. ●, subjects who were HLA-DR3$^+$ and/or HLA-DR4$^+$; □, subjects who were neither DR3$^+$ nor DR4$^+$. T1DM, type 1 diabetes.

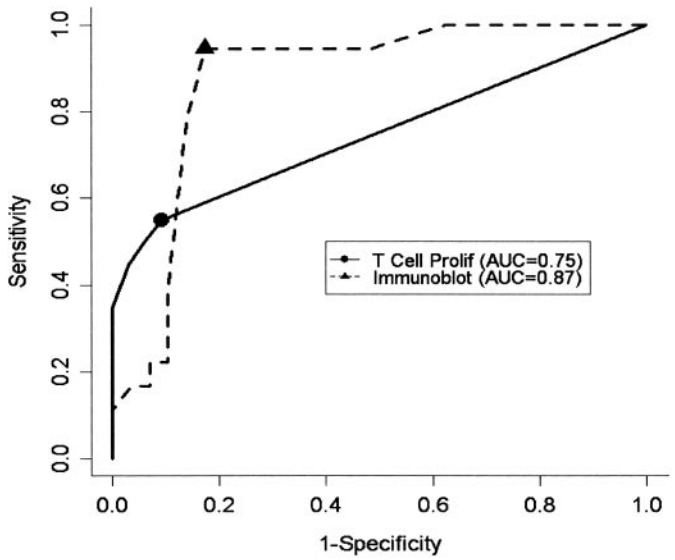

FIG. 2. Cellular assay ROC curves. The chosen thresholds for the immunoblot and T-cell proliferation assays offer optimal tradeoff between sensitivity and specificity based on the ROC curves; ROC curves were drawn based on the available data from this study. For the T-cell proliferation assay, a positive value was considered a score >3. Assays with a value of 3 were considered indeterminate, and values <3 were considered negative. For the immunoblot assay, positive assays were defined as >3. Values <3 were considered negative (see RESEARCH DESIGN AND METHODS). AUC, area under the curve.

DR3$^+$ and/or DR4$^+$. When the analysis was limited to just DR3$^+$ and/or DR4$^+$ subjects, 53% of the subjects with type 1 diabetes were positive compared with 9% (2 of 22) of the healthy control subjects ($P < 0.01$).

Autoantibody detection. Of 23 individuals with type 1 diabetes, 18 were positive for at least one of the two autoantibodies (GAD65 or ICA512/IA2): 7 (30%) were positive for one autoantibody, and 11 (48%) were positive for two autoantibodies (Table 3 and Fig. 3). The frequency of positivity for the two autoantibodies was similar to that previously reported (25,26). Of 39 normal control subjects, 6 were also positive in the autoantibody assays, the majority (5 of 7) was positive for GAD65 alone and 1 was positive for both GAD65 and ICA512. In these control subjects, the positive autoantibodies were of low titer (Fig. 3).

TABLE 3
Results of autoantibody measurements among type 1 diabetic and healthy control participants*

	Patients with type 1 diabetes	Healthy control subjects
Neither	5	33
GAD65 only	6	5
ICA512 only	1	0
Both autoantibodies	11	1

Data are *n*. *$P < 0.001$ subjects with type 1 diabetes vs. healthy control subjects.

Individual and combined assay sensitivity and specificity. Reported analyses were performed using all available data, excluding the low-yield samples; a post hoc analysis including only the 41 subjects with complete data yielded similar classification probabilities. The cellular immunoblot correctly classified seven type 1 diabetic participants misclassified by the T-cell proliferation assay and one considered indeterminate (Fig. 4). The combined data analysis suggests that the cellular assays measure different responses, both of which identify individuals with type 1 diabetes (Table 4). A positive result in both assays had a sensitivity of 53% and a specificity of 94%; a positive result in either assay had a sensitivity of 95% and specificity of 78%. The specificity was improved to 100% when a positive cellular response was combined with a positive serological result, although the sensitivity dropped to 75%.

DISCUSSION

Although type 1 diabetes is believed to result from the cell-mediated destruction of β-cells, it has been difficult to establish assays that can measure the autoreactive cellular responses in peripheral blood from subjects with the disease. As a first step for developing assays that can monitor disease-associated immune responses, we evaluated, in a blinded controlled study, whether responses in individuals with type 1 diabetes can be distinguished from responses in normal control subjects. We have shown that T-cell responses to defined autoantigens or to islet proteins separated by electrophoresis can be used to differentiate responses in patients with type 1 diabetes and normal control subjects, and, when combined with measurements of standard biochemical autoantibodies, they can discriminate responses with a high degree of sensitivity and specificity. This finding indicates that there are sufficient numbers of T-cells in the peripheral blood, reactive with islet and other diabetes-associated antigens, to distinguish normal control subjects from those with type 1 diabetes. Although we do not have information on the phenotype of the cellular responses to antigens in these cellular assays, our finding of a quantitative difference in response is somewhat different from earlier findings by Arif et al. (27), who reported qualitative differences (i.e., exclusively IL-10 production in normal control subjects compared with γ-interferon and IL-10 responses in patients with type 1 diabetes) in responses to proinsulin peptides by ELISpot. It remains unknown, however, whether the responses that we found in the peripheral blood reflect the responses in the islets and/or draining pancreatic lymph nodes.

Our study design involved blinded samples from multiple different centers, and therefore the results reflect the performance of these studies in an unbiased manner. Each of the assays evaluated different responses to islet antigens, and therefore it is not surprising that by combining the assays, the specificity can be improved. Each of these assays may be useful for mechanistic studies of immune therapies in which the effects of the intervention on the responses that differentiate normal subjects and those with type 1 diabetes are of interest and for differentiating effects on cellular and humoral immune responses associated with the disease.

Biochemical autoantibody measurements have become the mainstay for identifying autoimmune disease in patients with diabetes (28). The threshold for a positive response in these assays has been set to have a false-positive rate of <1% in normal control subjects, and previous DASP workshops have shown that the median adjusted sensitivity for anti-GAD antibody was 84% and for ICA512 was 58%; all GAD65 and ICA512 assays found significant differences between patients and control subjects in that workshop (29,30). In our study, either autoantibody alone scored well, with a sensitivity of 78% and a specificity of 85%, but the false-positive rate among healthy control subjects was higher than expected. Our results are surprising and significantly different from the previous performance of this assay in DASP workshops. One-half of the subjects with false-positive autoantibodies were HLA $DR3^+$ or $DR4^+$, and one-half were not, suggesting that HLA genotype was not a determinant of the false-positive findings. Although the normal control subjects did not have type 1 diabetes at the time of study, we do not have prospective data to be certain that the control subjects with two autoantibodies did not progress to diabetes. After completion of this study, the eight samples were reassayed. Six of the seven GAD65 samples did confirm positive, but one did not (original 0.095, repeat 0.029, and second repeat 0.021, with a positive cutoff of 0.032). The ICA512 repeated as positive. Because, in all but one case, the positive finding was reconfirmed in the control samples, this unexpectedly high rate of positive findings in the control group likely reflects an unusual set of normal control subjects given the small dataset. We cannot exclude as a contributing factor the possibility that samples were switched, since the individuals were not available for retesting.

Overall, the T-cellular responses performed well, with sensitivities of 94 and 58%, but specificities that were lower might be considered optimal in assessing changes in responses after treatment with immune-modulatory agents. The association of DR3 or DR4 alleles with a false-positive response in the immunoblot assay raises the possibility that some of the responses that were observed may be related to allogeneic differences between the islet-derived tissue and the responder. The islets were derived from multiple normal donors, but the HLA frequency in the donors is more akin to the normal control subjects than the patients with type 1 diabetes. However, allogeneic responses cannot account for the differences between the

TABLE 4
Sensitivity and specificity of combined assays

	n	Sensitivity	Composite 95% CI	Specificity	Composite 95% CI
Both cellular assays	52	0.53	0.29–0.76	0.94	0.86–1.0
Either cellular assays	47	0.95	0.85–1.0	0.78	0.63–0.93
Both cellular assays and either autoantibody	57	0.35	0.12–0.59	1.0	0.92–1.0
Either cellular assay and either autoantibody	58	0.75	0.55–0.90	1.0	0.92–1.0
Either autoantibody	62	0.78	0.61–0.96	0.85	0.72–0.95
Both autoantibodies	62	0.48	0.26–0.70	0.97	0.92–1.0

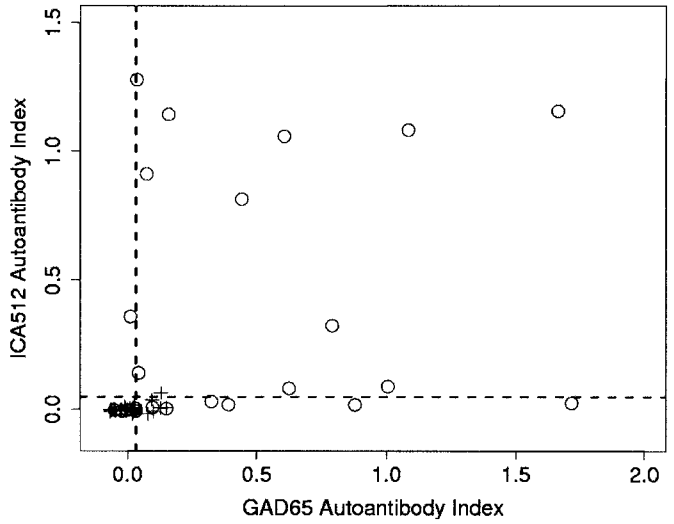

FIG. 3. Autoantibody indexes in study subjects. Serum samples were collected from subjects with type 1 diabetes and normal control subjects and analyzed for anti-GAD65 and anti-ICA512 autoantibodies. The false-positive results in normal control subjects showed low indexes. The dashed line is the threshold. ○, samples from subjects with type 1 diabetes; +, samples from normal control subjects.

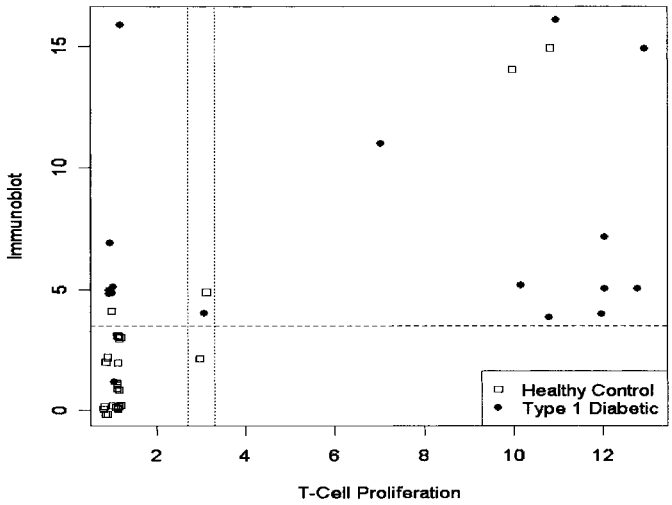

FIG. 4. Relationship between results in the two cellular assays by diabetes status. The results for each individual in the two cellular assays are plotted. Values above the dashed line were designated as positive, and values between the two dotted lines were designated as indeterminate and not used in the statistical analysis.

groups because there was a significantly greater response in the subjects with type 1 diabetes, even when the analysis was restricted to those with diabetes-associated class II alleles. In future studies, involving larger samples, matching for HLA would improve the statistical power to address this issue.

Our finding that two of the false positives were seen in both cellular assays suggests that it represents a true cellular response to islet antigens, even though neither of these subjects had a positive autoantibody response. This finding is not entirely unexpected because Arif et al. (27) have previously shown that there are positive ELISpot responses to proinsulin peptides in normal control subjects, but the phenotype of the responses is different from subjects with type 1 diabetes. We did not determine the cytokine responses to islet antigens in these assays, but they may provide additional insight into the nature of the responses.

By combining the assay results, we were able to improve the specificity and maintain sensitivity for identifying responses in subjects with type 1 diabetes. We show that the true positives are increased and false-positives lowered when combining information from multiple assays. The best combination of assays for discerning true positives and maximizing specificity was to classify a subject as positive if at least one cellular assay and at least one of the GAD65 or ICA512 autoantibody tests was positive. Most previous immune intervention trials in type 1 diabetes have not identified immune markers that can be used as biologic correlates of response associated with this T-cell–mediated disease. For example, in our studies of anti-CD3 monoclonal antibody treatment of patients with type 1 diabetes or trials of cyclosporin A in new-onset type 1 diabetes, there was no significant change in the titer or even the isotypes of autoantibodies with response (8,9). Studies of cellular responses, which are targeted by these interventions, have not been performed.

Reliable measurements of T-cell responses in subjects with type 1 diabetes have been problematic. In the first international workshop for standardization of T-cell assays in type 1 diabetes, centers were unable to discriminate responses of normal subjects from those of type 1 diabetic subjects (10). There are likely to be several reasons for these difficulties, including variability and impurities in antigen preparations, use of fresh versus frozen cells, and others. In addition, Dosch et al. (14) have shown general nonresponsiveness of cells from patients with type 1 diabetes, hence the need to supplement cultures with IL-2 or -15, as was done in this study. The use of frozen PBMCs allows comparison between multiple time points in the same assay but has additional technical problems: the two assays in our study used fresh cells and antigen preparations that were made by each laboratory.

There are a number of limitations of the approaches that were studied. First, in the immunoblot assay, it is not clear to which antigens the responses are directed and whether all subjects with type 1 diabetes respond to the same or different antigens. In future assays, adding tissue controls, for example, might improve the sensitivity and specificity of the immunoblot assay. Second, the assays depend on expansion of the cells in vitro so that both the proliferative responses as well as the number of antigen-reactive T-cell precursors affect the final assay result. The absence of a response in vitro could be caused by elimination of antigen-reactive cells, regulation of the antigen-specific cells by induced regulatory T-cells, changes in cytokine production, or other mechanisms. Nonetheless, because the frequency of $CD4^+$ cells that are reactive to any single antigen is very low, it is unlikely that these cells could be enumerated without expansion. Finally, the starting material for these cellular assays is large, which would limit their usefulness for smaller subjects or for repeated testing.

It will also be important to define the antigen specificity of the responses measured in the cellular assays to understand the significance of these responses in the disease process. It has already been reported that PBMCs from multiple sclerosis patients also respond to the GAD peptides in the T-cell proliferation assay (23). Interestingly, reactivity of T-cells from multiple sclerosis patients also have been shown to score positive in the immunoblot assay, using whole-islet antigen preparations (B.B.-W., unpublished observations). It is possible that the cross-

reactivity observed in these assays actually reflects differences in T-cell activation thresholds between autoimmune versus healthy individuals in a non–antigen-specific manner. This difference may be augmented by the assay conditions, such as the exogenously added IL-2 and -15 or whatever other components are in the whole-islet preparations.

In summary, in a blinded controlled study, we have shown that the combination of cellular assays and autoantibody measurements can discriminate immune responses in subjects with type 1 diabetes from those in healthy control subjects. False-positive results were seen in all three of the immunoassays but in none of the subjects when the assays were combined. The use of these assays will provide important information about disease-associated cellular responses that may prove useful in future studies of immune interventions. Further studies are needed to provide qualitative information about the response, identify antigens recognized in whole-islet preparations, and reduce the volume of sample needed.

ACKNOWLEDGMENTS

This study was supported by a subcontract from the ITN, a project of the National Institute of Allergy and Infectious Diseases; the National Institute for Diabetes and Digestive and Kidney Diseases; and the Juvenile Diabetes Research Foundation.

REFERENCES

1. Atkinson MA, Eisenbarth GS: Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 358:221–229, 2001
2. Atkinson MA, Maclaren NK: The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med* 331:1428–1436, 1994
3. Roep BO: The role of T-cells in the pathogenesis of type 1 diabetes: from cause to cure. *Diabetologia* 46:305–321, 2003
4. Riley WJ, Maclaren NK, Krischer J, Spillar RP, Silverstein JH, Schatz DA, Schwartz S, Malone J, Shah S, Vadheim C, et al.: A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. *N Engl J Med* 323:1167–1172, 1990
5. Yu L, Rewers M, Gianani R, Kawasaki E, Zhang Y, Verge C, Chase P, Klingensmith G, Erlich H, Norris J, Eisenbarth GS: Antiislet autoantibodies usually develop sequentially rather than simultaneously. *J Clin Endocrinol Metab* 81:4264–4267, 1996
6. Eisenbarth GS: Type I diabetes mellitus: a chronic autoimmune disease. *N Engl J Med* 314:1360–1368, 1986
7. Eisenbarth GS: Insulin autoimmunity: immunogenetics/immunopathogenesis of type 1A diabetes. *Ann N Y Acad Sci* 1005:109–118, 2003
8. Herold KC, Hagopian W, Auger JA, Poumian-Ruiz E, Taylor L, Donaldson D, Gitelman SE, Harlan DM, Xu D, Zivin RA, Bluestone JA: Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. *N Engl J Med* 346:1692–1698, 2002
9. Bougneres PF, Carel JC, Castano L, Boitard C, Gardin JP, Landais P, Hors J, Mihatsch MJ, Paillard M, Chaussain JL, et al.: Factors associated with early remission of type I diabetes in children treated with cyclosporine. *N Engl J Med* 318:663–670, 1988
10. Roep BO, Atkinson MA, van Endert PM, Gottlieb PA, Wilson SB, Sachs JA: Autoreactive T cell responses in insulin-dependent (type 1) diabetes mellitus: report of the first international workshop for standardization of T cell assays. *J Autoimmun* 13:267–282, 1999
11. Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK: Cellular immunity to a determinant common to glutamate decarboxylase and Coxsackie virus in insulin-dependent diabetes. *J Clin Invest* 94:2125–2129, 1994
12. Ellis T, Jodoin E, Ottendorfer E, Salisbury P, She JX, Schatz D, Atkinson MA: Cellular immune responses against proinsulin: no evidence for enhanced reactivity in individuals with IDDM. *Diabetes* 48:299–303, 1999
13. Ellis TM, Schatz DA, Ottendorfer EW, Lan MS, Wasserfall C, Salisbury PJ, She JX, Notkins AL, Maclaren NK, Atkinson MA: The relationship between humoral and cellular immunity to IA-2 in IDDM. *Diabetes* 47:566–569, 1998
14. Dosch H, Cheung RK, Karges W, Pietropaolo M, Becker DJ: Persistent T cell anergy in human type 1 diabetes. *J Immunol* 163:6933–6940, 1999
15. Reijonen H, Kwok WW: Use of HLA class II tetramers in tracking antigen-specific T cells and mapping T-cell epitopes. *Methods* 29:282–288, 2003
16. Anthony DD, Lehmann PV: T-cell epitope mapping using the ELISPOT approach. *Methods* 29:260–269, 2003
17. Brooks-Worrell BM, Starkebaum GA, Greenbaum C, Palmer JP: Peripheral blood mononuclear cells of insulin-dependent diabetic patients respond to multiple islet cell proteins. *J Immunol* 157:5668–5674, 1996
18. Brooks-Worrell B, Gersuk VH, Greenbaum C, Palmer JP: Intermolecular antigen spreading occurs during the preclinical period of human type 1 diabetes. *J Immunol* 166:5265–5270, 2001
19. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
20. Abou-Zeid C, Filley E, Steele J, Rook GA: A simple new method for using antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocytes in vitro after converting bands cut from Western blots into antigen-bearing particles. *J Immunol Methods* 98:5–10, 1987
21. Miyazaki I, Cheung RK, Gaedigk R, Hui MF, Van der Meulen J, Rajotte RV, Dosch HM: T cell activation and anergy to islet cell antigen in type I diabetes. *J Immunol* 154:1461–1469, 1995
22. Winer S, Astsaturov I, Cheung RK, Schrade K, Gunaratnam L, Wood DD, Moscarello MA, O'Connor P, McKerlie C, Becker DJ, Dosch HM: T cells of multiple sclerosis patients target a common environmental peptide that causes encephalitis in mice. *J Immunol* 166:4751–4756, 2001
23. Winer S, Astsaturov I, Cheung R, Gunaratnam L, Kubiak V, Cortez MA, Moscarello M, O'Connor PW, McKerlie C, Becker DJ, Dosch HM: Type I diabetes and multiple sclerosis patients target islet plus central nervous system autoantigens; nonimmunized nonobese diabetic mice can develop autoimmune encephalitis. *J Immunol* 166:2831–2841, 2001
24. Winer S, Tsui H, Lau A, Song A, Li X, Cheung RK, Sampson A, Afifiyan F, Elford A, Jackowski G, Becker DJ, Santamaria P, Ohashi P, Dosch HM: Autoimmune islet destruction in spontaneous type 1 diabetes is not beta-cell exclusive. *Nat Med* 9:198–205, 2003
25. Eisenbarth GS, Gianani R, Yu L, Pietropaolo M, Verge CF, Chase HP, Redondo MJ, Colman P, Harrison L, Jackson R: Dual-parameter model for prediction of type I diabetes mellitus. *Proc Assoc Am Physicians* 110:126–135, 1998
26. Yu L, Cuthbertson DD, Maclaren N, Jackson R, Palmer JP, Orban T, Eisenbarth GS, Krischer JP: Expression of GAD65 and islet cell antibody (ICA512) autoantibodies among cytoplasmic ICA+ relatives is associated with eligibility for the Diabetes Prevention Trial-Type 1. *Diabetes* 50:1735–1740, 2001
27. Arif S, Tree TI, Astill TP, Tremble JM, Bishop AJ, Dayan CM, Roep BO, Peakman M: Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J Clin Invest* 113:451–463, 2004
28. Zamaklar M, Jotic A, Lalic N, Lalic K, Rajkovic N, Milicic T: Relation between course of disease in type 1 diabetes and islet cell antibodies. *Ann N Y Acad Sci* 958:251–253, 2002
29. Bingley PJ, Bonifacio E, Mueller PW: Diabetes Antibody Standardization Program: first assay proficiency evaluation. *Diabetes* 52:1128–1136, 2003
30. Mueller PW, Bingley PJ, Bonifacio E, Steinberg KK, Sampson EJ: Predicting type 1 diabetes using autoantibodies: the latest results from the Diabetes Autoantibody Standardization Program. *Diabetes Technol Ther* 4:397–400, 2002