Detection of GAD65-Reactive T-Cells in Type 1 Diabetes by Immunoglobulin-Free ELISPOT Assays

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OBJECTIVE — To investigate the prevalence of β-cell autoantigen-reactive peripheral T-cells in type 1 diabetes, we developed an immunoglobulin-free enzyme-linked immunospot (ELISPOT) assay and assessed its usefulness for diagnosing this disease.

RESEARCH DESIGN AND METHODS — Cellular immune responses to β-cell autoantigens were studied both by immunoglobulin-free proliferation assays and ELISPOT assays in 33 patients with type 1 diabetes and 15 patients with type 2 diabetes, compared with 23 healthy control subjects. Antibodies against GAD65 and IA-2 were measured by radioimmunoassay.

RESULTS — Significant proliferative responses to GAD65 were observed in 10 of 31 (32.3%) type 1 diabetic patients (P < 0.05), whereas GAD65-reactive IFN-γ-secreting cells were detected in 22 of 33 patients (66.7%) by ELISPOT assay (P < 0.001). Of patients negative for both GAD65 and IA-2, five of six (83.3%) showed IFN-γ positivity in ELISPOT and two of five (40.0%) showed significant proliferation against GAD65.

CONCLUSIONS — Using a newly developed ELISPOT assay, GAD-reactive T-helper 1 cells in PBMC of type 1 diabetic patients could be identified at a higher frequency than by the proliferation assay. Therefore, the immunoglobulin-free ELISPOT assay is an excellent tool for detecting T-cell reactivity to autoantigens with greater specificity and, in combination with the proliferation assay, can identify a subset of latent autoimmunity in type 1 diabetes.

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Several studies of type 1 diabetes have suggested that the destruction of pancreatic β-cells is caused by inflammatory cellular immune responses mediated by T-helper 1 (Th1) cells, which secrete γ-interferon (IFN-γ) (1–3). In diabetic animal models, a correlation between disease onset and Th1 cytokine production has been observed, and IFN-γ in particular plays an important role in autoantibodies are considerably lower in patients with a long disease duration than in patients at the time of onset or in the prediabetic period (12). According to a Japanese multicenter study, positivity for GAD antibody was found in only 35.4% of 921 Japanese type 1 diabetic patients studied, but among those with newly diagnosed diabetes, 50.3% were GAD antibody positive (13). Japanese type 1 diabetic subjects, therefore, seemed to present with lower antibody levels than Caucasians. In addition, it has been suggested that the presence of GAD antibody can identify a subset of latent autoimmune diabetes in adults (LADA) and predict incipient insulin dependency (14,15). Indeed, the patient’s autoantibody status is essential for the diagnosis and prediction of type 1 diabetes but is not sufficiently definitive in Japanese patients.

Because autoreactive T-cells are assumed to be the key effector cells that destroy pancreatic β-cells, the demonstration of autoreactive T-cells could represent a good index of progressive β-cell destruction. Measurement of cellular immune reactivity against β-cell autoantigen may reflect a general increase in T-cell responsiveness, and therefore, may be superior to determining the level of autoantibodies to the same antigen for disease prediction. So far, the cellular immune response to β-cell antigens in humans has been evaluated by measuring proliferative responses of human peripheral blood mononuclear cells (PBMCs) by means of the [3H]thymidine incorporation assay. However, this technique is affected by many factors, including differences in autoantigen preparations and culture conditions, so that the published data on T-cell reactivity to various candidate autoantigens have been inconsistent (16,17). One approach to investigating cellular immune responses and the immunopathogenesis of diabetes in humans is to identify the cytokines secreted by PBMCs of type 1 diabetic patients, analyze their function, and correlate these with the patients’ profiles. Recently, studies...
have been undertaken using the solid-phase enzyme-linked immunosorbent ELISPOT assay, which is an easily performed test that offers a quick means of visualization of nitrocellulose-adsorbed substances and can readily be used for performing field epidemiological surveys when costly, sophisticated technology is lacking. On the assumption that immunoglobulin in the assay medium would cause nonspecific reactions in human T-cells, an immunoglobulin-free culture system was established for our T-cell assays. In the present study, we have measured the responses of pancreatic β-cell-reactive peripheral T-cells by using an immunoglobulin-free ELISPOT assay to identify secreted cytokines.

RESEARCH DESIGN AND METHODS

Subjects
A total of 33 Japanese type 1 diabetic patients (diagnosed according to the criteria of the American Diabetes Association) were studied (14 male patients aged 15–65 years and 19 female patients aged 5–75 years; mean ± SD, 32.7 ± 17.8 years). The clinical characteristics of these 33 patients are summarized in Table 1. A total of 15 patients with type 2 diabetes (six men aged 33–67 years and nine women aged 35–77 years; 53.9 ± 15.6 years) as well as 23 healthy volunteers without diabetes or any other autoimmune disease (10 men aged 26–42 years and 13 women aged 22–52 years; 32.2 ± 6.1 years) were included in the study. Serum antibodies to GAD and tyrosine phosphatase–like protein (IA-2) were measured by the Cosmic GAD antibody radioimmunoassay kit (Cosmic, Tokyo, Japan) and the IA-2 immunoprecipitation assay kit (Cosmic, respectively) (18,19).

Antigens
The soluble form of human recombinant GAD65 protein used in this study had the NH2-terminal amino acids from 2 to 45 inclusive deleted (18) (Cosmic, Tokyo). β-Casein from bovine milk was from Fluka Chemical (Ronkonkoma, NY). Phytohemagglutinin (PHA) was from Wako Chemicals (Osaka, Japan), and purified protein derivative (PPD) of Escherichia coli was from Nippon (Tokyo, Japan). The purity of GAD65 and β-casein was >95% and >90%, respectively. The content of endotoxin in 10 μg GAD65 was very low (0.035 endotoxin units).

Proliferation assay
Heparinized, freshly drawn blood samples from each subject were assayed within 12 h. PBMC were isolated by Ficoll-Paque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden); 2 × 10⁵ PBMC per well were cultured in round-bottomed, 96-well tissue culture plates at 37°C, 5% CO₂, in 200 μl of RPMI-1640 with 2 mmol/L L-glutamine, supplemented with 10% human type AB serum, 5 × 10⁻⁵ mol/L 2-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin (Flow Laboratories, McLean, VA). The medium was filtered through cellulose ester membrane (Spectrum Medical Industries, Houston, TX) to remove substances of >100,000 molecular weight (MW), including immunoglobulins, which were present thereafter at <2 μg/ml total IgG.

Stimulants in this study were used at an appropriate concentration as follows: 10 μg/ml GAD65, 10 μg/ml β-casein, 1 μg/ml PPD, 10 μg/ml PHA. After 6 days' culture, 1 μCi [³H]thymidine (20–30 Ci/mmol; Amersham, Tokyo, Japan) was added 16 h before harvesting. Cells were then harvested using a Packard FilterMate harvester (Packard, Meriden, CT), and [³H]thymidine incorporation was measured on a TopCount Microplate scintillation counter (Packard). The means of triplicate cultures of each subject and stimulant were determined, and the stimulation index (mean cpm in the presence of Ag/mean cpm without Ag) was calculated. Values are given as means of stimulation index (SD) ± SD. An SI greater than the mean + 2 SD of the healthy control population was considered to represent a positive response.

ELISPOT assay
Nitro-cellulose–bottomed, 96-well microtiter plates (Millititer; Millipore, Bedford, MA) were coated with 15 μg/ml mouse anti-human IFN-γ monoclonal antibody (mAb) (1-DIK; Mabtech AB, Stockholm, Sweden) or mouse anti-human interleukin-4 (IL-4) mAb (IL-4-1; Mabtech AB) in sterile coating buffer (0.1 mol/L carbonate-bicarbonate buffer, pH 9.6) overnight at 4°C. Unbound antibodies were removed by washing six times with sterile phosphate-buffered solution. Aliquots of 2 × 10⁵ PBMCs per well were incubated in mAb-coated plates with the same antigens/stimulators as in the proliferation assays under the same culture conditions for 40 h. After washing, 1 μg/ml biotin-conjugated anti-cytokine mAb (7-B6-1 for IFN-γ and IL-4-II for IL-4) was added to each well and incubated for 3 h at room temperature followed by streptavidin conjugated with alkaline phosphatase for 1 h. Finally, BCIP/NBT substrate solution (Bio-Rad Laboratories, Richmond, CA) was added and incubated until the appearance of blue spots in the wells (15–25 min). All plates were counted on the computer screen, viewing through a dissection microscope, always by the same investigator. The plates were counted by a blinded investigator to avoid any influence of knowledge of the contents on the outcome. Even though the spots varied greatly in size and density, homogeneously stained spots were seen in positive wells, whereas the small dense spots that were occasionally seen both in wells with cells and in control wells without cells were distinguished as artifacts by using a National Institutes of Health imager with a charge-coupled device camera. All data are expressed as means of triplicate determinations for each antigen and lymphocyte concentration. The number of spots obtained as a result of antigen stimulation was determined as [(mean number of spots in the presence of antigen) – (mean obtained without stimulation)] and referred to here as “antigen-stimulated.” When the number of spots was greater than the mean + 3 SD of the controls or the nonresponders, this was considered a positive response.

Statistical analysis
For the proliferation and ELISPOT assays, two groups were compared using the Mann-Whitney U test, and three groups
were compared with the Kruskal-Wallis test. Spearman’s correlation coefficient was used when comparing two nonparametric variables. The \(H^2\) test was used to compare T-cell response frequencies among the groups. For cases in which the expected values were <5, Fisher’s exact test was used. One-way factorial ANOVA and multiple comparison tests were used to compare the ranges of the proliferative responses to positive controls (PPD and PHA) among the groups. \(P\) values <0.05 were deemed statistically significant.

**RESULTS**

**Level of responsiveness in the immunoglobulin-free proliferation assay**

To avoid any antigen-nonspecific T-cell stimulation, we established an immunoglobulin-free culture system in which molecules of \(>100,000\) MW were excluded from the culture media. PHA was used as a positive control to confirm that this immunoglobulin-free technique was suitable for the T-cell assay. Such mitogenic responses were consistently high in all patients with type 1 and type 2 diabetes as well as in the control group (unstimulated background was 582 ± 714, 550 ± 1,096, and 791 ± 948 cpm, respectively, and SI was 93.4 ± 41.3, 126.0 ± 99.2, and 84.9 ± 56.5, respectively). No significant differences were observed for PHA responsiveness in these three groups (Table 1). Additionally, PPD was used as a Th1-directed positive control antigen in this study because it is well known to elicit a Th1-dominant immune response.

**Table 1—Profile of patients and results of proliferation and ELISPOT assays**

<table>
<thead>
<tr>
<th>Subject (initial)</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Duration of diabetes (years)</th>
<th>IA-2 (units/ml)</th>
<th>GAD (units/ml)</th>
<th>PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control subjects (n = 23)</td>
<td>F</td>
<td>32.2 ± 6.1</td>
<td></td>
<td>84.9 ± 56.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 2 diabetes (n = 15)</td>
<td>F</td>
<td>53.9 ± 13.6</td>
<td></td>
<td>126.0 ± 99.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1 diabetes (n = 33)</td>
<td>M</td>
<td>32.7 ± 17.8</td>
<td>7.3 ± 9.0</td>
<td>93.4 ± 41.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject (initial)</th>
<th>Sex</th>
<th>Autoantibody</th>
<th>IA-2 (units/ml)</th>
<th>GAD (units/ml)</th>
<th>PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control subjects (n = 23)</td>
<td>F</td>
<td>IA-2</td>
<td>84.9 ± 56.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 2 diabetes (n = 15)</td>
<td>F</td>
<td>GAD</td>
<td>126.0 ± 99.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1 diabetes (n = 33)</td>
<td>M</td>
<td>PHA</td>
<td>93.4 ± 41.3</td>
<td></td>
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</tr>
</tbody>
</table>

Data are means ± SD. *\(P\) < 0.05 when compared with three groups (Kruskal-Wallis test).
response in most Japanese patients. Proliferative responses to PPD varied over an SI range from 1.4 to 409.1, and no significant differences in responsiveness were observed among the three responding groups.

**T-cell proliferative responses to β-cell autoantigens**

Simultaneous lymphocyte proliferative responses against GAD65 and β-casein are shown in Fig. 1A. With respect to stimulation by GAD65, 10 of 31 (32.3%) patients with type 1 diabetes had a positive response, whereas none of the patients with type 2 diabetes or control subjects responded to GAD65 ($P < 0.05$). In patients with recent-onset type 1 diabetes, 4 of 13 (30.8%) showed positive proliferative responses, and in patients with established type 1 diabetes, this value was 33.3% (6 of 18 patients) (Table 1). This suggests that T-cell responses against GAD65 persist for an extended period after onset of diabetes. Proliferative responses to β-casein were observed in 6 of 29 (20.7%) patients with type 1 diabetes compared with only 1 of 12 (8.3%) patients with type 2 diabetes and none of the 20 healthy control subjects.

**Positive and negative controls for the immunoglobulin-free ELISPOT assay**

In the ELISPOT assay using immunoglobulin-free media, 45 of 71 (63.4%) subjects in this study produced fewer than one IFN-γ spot per $2 \times 10^5$ PBMCs and only five subjects (7.0%) produced more than five IFN-γ spots, in the absence of any stimulatory agents. Therefore, the
mean number of IFN-γ spots in wells containing only T-cells was 1.0 ± 2.2 per 2 × 10^5 PBMCs, indicating little nonspecific IFN-γ production. In contrast, no IL-4 spots were detected at all in any wells without stimulation. Therefore, nonspecific spots in the absence of antigen were seldom seen in the ELISPOT assay using immunoglobulin-free media. On the other hand, PHA stimulation always generated large numbers of IFN-γ and IL-4 spots (>100 per well) in all three groups. Stimulation with PPD resulted in different numbers of IFN-γ spots, depending on the donor (0–50 per 2 × 10^5 PBMC), whereas no IL-4 spots were detected in either patients with diabetes or control subjects.

**β-cell antigen–reactive T-cells in the ELISPOT assay**

PBMCs from 22 of 33 (66.7%) type 1 diabetic patients yielded positive GAD-stimulated IFN-γ spots after subtraction of the number of spots in the unstimulated culture, whereas only 1 of 15 patients with type 2 diabetes (6.7%) and none of the 23 healthy control subjects had ≥0.8 spot (P < 0.001, Fig. 1B). Among 10 patients with type 1 diabetes who had positive proliferative responses to GAD, 7 (70%) yielded IFN-γ spots in this assay. On the other hand, 6 of 33 patients with type 1 diabetes generated significant numbers of IL-4 spots (P < 0.05), and of 6 IL-4–positive patients with type 1 diabetes, 5 also showed positivity for IFN-γ spots as well (Table 1).

In response to β-casein, 9 of 25 (36.0%) patients with type 1 diabetes yielded more than one IFN-γ spot, similar to the level found in control subjects (26.3%; 5 of 19 subjects). In type 2 diabetes, only one patient generated β-casein–stimulated IFN-γ spots. β-Casein failed to stimulate any IL-4 spots in either diabetic patients or normal control subjects. Among six patients with type 1 diabetes who had positive proliferative responses to β-casein, only one (subject 12) yielded β-casein–stimulated IFN-γ spots; the remainders were negative.

**Significance of combined T-cell assays in autoantibody-negative patients**

Among 30 patients with type 1 diabetes in whom both GAD and IA-2 were assessed, it was found that 11 were positive for both autoantibodies, 13 were positive for only one autoantibody, and 6 for neither autoantibody. Interestingly, GAD-stimulated IFN-γ spots were detected in five of six (83.3%) patients who were negative for both GAD and IA-2 autoantibodies. In contrast, proliferative responses to GAD were observed in only two of five (40.0%) autoantibody-negative patients. In the present study, all except one patient with type 1 diabetes had a positive response according to at least one of the three indexes: GAD autoantibody, IA-2 autoantibody, or ELISPOT assay positivity. The single autoantibody-negative and T-cell response–negative patient was a 20-year-old woman with onset of diabetes at 5 years of age (subject 25). Lack of detectable autoimmunity in this patient may be accounted for by the extended period after onset or possibly by nonautoimmune type 1 diabetes in this instance. In conclusion, combining the detection of autoan-
tibodies with T-cell assays is useful for the detection of autoimmune processes.

CONCLUSIONS — Recent studies have demonstrated that the pathogenesis of type 1 diabetes is mainly due to a T-cell–mediated autoimmune process, leading to the complete destruction of pancreatic β-cells. In routine clinical analysis, however, islet cell antibodies, autoantibodies against GAD, insulin, and IA-2 are usually used as markers for diagnosis of autoimmune type 1 diabetes (7–9). This may be partly because of the difficulties in establishing a reliable internationally accepted, antigen-specific diagnostic T-cell assay (16,17). We hypothesized that one of the factors contributing to irreproducibility of human T-cell assays is the requirement for the presence of human serum in the culture medium.

In previous studies using the ELISPOT assay, 10–100 spots were commonly detected even in nonstimulated negative controls, compared with >100 spots in antigen-stimulated wells; this seemed to be an unacceptably high background (20,21). These observations suggested to us that nonspecific stimulation under antigen-free conditions might be due to immunoglobulin in the media. To eliminate any such substances, a membrane that excludes molecules of >100,000 MW was used to prepare the medium for T-cell culture. In a preliminary study using five patients with type 1 diabetes, we performed both proliferation and ELISPOT assays in culture medium with untreated human AB serum compared with immunoglobulin-free AB serum in which molecules of >100,000 MW were excluded. For proliferative response against GAD, the mean SI in immunoglobulin-free medium (8.5 ± 3.3) was higher than in medium with unseparated serum (3.5 ± 2.0). Furthermore, the SI decreased (to 2.8 ± 2.5) when purified human IgG (1 mg/ml) was added back to immunoglobulin-free media, indicating that immunoglobulins might have some adverse effects on [3H]thymidine incorporation. In the ELISPOT assay, the number of IFN-γ spots was significantly lower in unstimulated control wells in immunoglobulin-free medium (mean 0.9 ± 1.4), compared with medium with complete serum (16.2 ± 15.0). In the presence of GAD, more IFN-γ spots (23.0 ± 18.0) were detected using medium supplemented with unseparated serum than in immunoglobulin-free medium (3.7 ± 3.3). After subtraction of the background number of spots in the unstimulated cultures, there seemed to be more GAD-stimulated IFN-γ spots in medium with full serum (6.8 ± 4.1), but this did not reach significance compared with unstimulated spots (Mann-Whitney U test; P = 0.20). In contrast, even though the absolute number seemed small after subtraction of background (2.8 ± 2.3) under immunoglobulin-free conditions, this value was significantly different compared with the number of unstimulated spots (P = 0.047). In this study, GAD-stimulated IFN-γ production in immunoglobulin-free medium in type 1 diabetes yielded 2.9 ± 4.6 spots per 2 × 10^5 PBMCs, apparently higher than in healthy control subjects (0.1 ± 0.2, Student’s t test; P < 0.001) (Table 1). Although the cutoff value for GAD-stimulated IFN-γ spots was low (0.79 spots) and close to the values of three control subjects (0.67 spots), 22 patients with type 1 diabetes and 1 patient with type 2 diabetes showed positive GAD-stimulated IFN-γ spots more than two times higher than background. In nine control subjects with background spots, on the other hand, GAD-stimulated IFN-γ spots before subtraction were only an increase of <0.5 times the background. Therefore, removal of immunoglobulin from the medium can allow a small number of GAD-reactive T-cells to be distinguished from nonspecific reactivity. Furthermore, human antibodies in enzyme-linked immunosorbent assay media can bind to mouse antibodies, leading to false positives in human cytokine assays (22). Taken together, it is concluded that the level of background noise in the ELISPOT assay can be reduced by using immunoglobulin-free media, which can then reveal a small number of GAD-reactive T-cells.

Using this immunoglobulin-free ELISPOT assay, the frequency of detectable anti-GAD cellular immunity was 66.7% in patients with type 1 diabetes, which was markedly higher than the 32.3% established using the proliferation assay (P < 0.005; χ^2 test). On the other hand, some cases that proliferated vigorously with GAD did not show any apparent IFN-γ or IL-4 spots. This might be explained by the fact that PBMC proliferation reflects the responses not only of T-cells but also of other immune cells such as natural killer T-cells.

One of the problems with the immunoglobulin-free ELISPOT assay is whether the lower number of positive cytokine spots in the antigen-stimulated culture reflects the exact number of antigen-specific cells. The frequency of spots (1–23 per 200,000 cells) implied a frequency of GAD-reactive T-cells of 1.200,000~1.10,000. In patients with multiple sclerosis, the frequency of antigen-specific T-cells has been reported as 1 in 10^5–10^6 T-cells, when measured by limiting dilution analysis (23–25). Biegansowska et al. (26), however, estimated frequencies of T-cells expressing specific autoimmune peptide–associated T-cell receptor chain transcripts to be as high as 1 in 300, and proposed that Fas-mediated cell death upon antigen stimulation was responsible for the low measured frequency of autoreactive T-cells in the blood of patients. Similarly, the low frequency of antigen-reactive IFN-γ spots detected by ELISPOT assay in type 1 diabetes in the present study may reflect activation-induced cell death of in vivo activated, autoreactive T-cells in the in vitro antigen-stimulated assay cultures. In addition, our complete removal of immunoglobulin might also have affected the T-cell response. It has been reported that serum GAD65 autoantibodies enhance the anti-islet response in a human GAD-specific T-cell hybridoma (27). Taken together, activation-induced cell death and autoantibodies may affect the immunoglobulin-free ELISPOT assay, resulting in a relatively low number of reactive spots.

GAD-IFN-γ ELISPOT assays were repeated in four patients with type 1 diabetes (subjects 11, 12, 27, and 30) who showed positive GAD-stimulated IFN-γ spots (10.0, 23.0, 4.0, and 13.0, respectively). All patients consistently showed significant GAD-stimulated IFN-γ spots (8.0, 19.0, 2.7, and 15.3, respectively) on the second occasion as well. Additionally, ELISPOT assays were performed on three frozen samples (subjects 11, 12, and 30) that had been kept for ~1 month. Similar positive results were obtained on all of them (IFN-γ spots: 6.7, 15.3, and 8.0, respectively). This suggests that the immunoglobulin-free ELISPOT assay is a very reproducible method. In addition, the ELISPOT assay using frozen samples is a useful tool for monitoring T-cell responses of subjects in intervention trials.
Detection of GAD65-reactive T-cells in type 1 diabetes

The other potential autoantigen tested here, β-casein purified from bovine milk, was investigated because of the cow’s milk hypothesis for the cause of type 1 diabetes (28,29). However, normal healthy control subjects also produced more than one IFN-γ spot. The reason for this remains unclear. Because previous studies were on Caucasian subjects, it is possible that type 1 diabetes is not correlated with a high prevalence of β-casein-reactive, IFN-γ-secreting T-cells in Japanese individuals.

In conclusion, ELISPOT assays can detect β-cell–reactive Th1 cells in PBMC of patients with type 1 diabetes at a higher frequency and with less false-positives when performed immunoglobulin-free. By combining autoantibody and this ELISPOT assay, autoimmunity can be identified in 97% (32 of 33) of patients with type 1 diabetes. One patient with type 2 diabetes showed GAD seronegativity but positive GAD-stimulated IFN-γ spots. Type 2 diabetes was diagnosed in this patient 10 years prior, but his insulin secretion was relatively low (fasting C-peptide response 0.22 nmol/l, C-peptide response 6 min after glucagon loading 0.56 nmol/l). It is possible that type 1 diabetes may yet develop in this patient. It is anticipated that the ELISPOT assay will be fully standardized in the near future and will provide a useful tool for diagnosis and prediction of type 1 diabetes.

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