Characterization of Preparations of GAD65, Proinsulin, and the Islet Tyrosine Phosphatase IA-2 for Use in Detection of Autoreactive T-Cells in Type 1 Diabetes

Report of Phase II of the Second International Immunology of Diabetes Society Workshop for Standardization of T-cell Assays in Type 1 Diabetes

Mark Peakman,1 Timothy I. Tree,1 Josef Endl,2 Peter van Endert,3 Mark A. Atkinson,4 and Bart O. Roep5

The identification, quantification, and characterization of T-cells reactive with the islet autoantigens GAD65, proinsulin (PI), and tyrosine phosphatase–like molecules IA-2 and phogrin are major research goals in type 1 diabetes. In the Immunology of Diabetes Society First Workshop on Autoreactive T-Cells, the quality of recombinant preparations of these autoantigens was identified as a significant weakness, a finding that may account for much of the inconsistency in published studies of peripheral blood T-cell reactivity to islet autoantigens. Poor antigen quality has also hampered the development of novel technologies for the detection of islet-reactive T-cells. For these reasons, in the present study, several preparations of GAD65, PI, and IA-2 were collected and evaluated for endotoxin content, ability to stimulate a panel of relevant T-cell clones, and inhibitory effects on proliferation to unrelated third-party antigens. Through this process, we have been able to identify preparations of GAD65 and IA-2, generated in insect cells using the baculovirus expression system, that stimulate relevant clones and display low inhibitory effects on third-party antigens. In addition, we characterized a PI preparation generated in bacteria as being free of effects on proliferation to third-party antigens and low in endotoxin content. These preparations are important to promote the development of robust and sensitive assays of islet-reactive T-cells in patients with type 1 diabetes or patients at high risk for developing the disease. Diabetes 50:1749–1754, 2001

Type 1 diabetes is considered to be an autoimmune disease in which islet antigen-reactive T-cells are involved in the destruction of insulin-producing β-cells (1). Currently, most of the evidence for this assertion remains circumstantial. Such evidence includes the characteristic T-cell–dominated islet infiltrate seen at diagnosis (2) and after pancreas transplantation between identical twins (3), the effects of immunosuppressive agents on disease progression (4), and case reports of type 1 diabetes development after “adoptive transfer” of diabetogenic T-cells during bone marrow transplantation from a diabetic individual to a related immuno-incompetent recipient (5).

Given this background, it is frustrating that there is a lack of consistency in reports on detection of islet autoreactive T-cells obtained from the peripheral blood of patients with type 1 diabetes or individuals at increased risk for the disease (6). The existence of such T-cells is evidenced by several reports of CD4+ T-cell clones (TCCs) generated from the peripheral blood and reactive against the major islet autoantigens (7–10). Further indirect evidence is provided by the well-documented existence of class-switched islet autoantigen–specific IgG class autoantibodies in >90% of type 1 diabetic patients at diagnosis (6), strongly implying the presence of T-cell–directed help. There are numerous possible explanations for the inconsistent nature of reports on autoreactive T-cells, including the probable low precursor frequency of circulating autoreactive T-cells, the inability to access lesional T-cells, and the potential presence of regulatory T-cells (11), as well as the varied characteristics of the autoantigens and techniques used.

It is important that research on T-cell autoreactivity in type 1 diabetes is able to progress. Autoreactive T-cells may hold clues to the mechanism of diabetes development and offer potentially important surrogate markers of islet cell damage for use in therapeutic trials (12).

To examine these issues in more detail, the Immunology of Diabetes Society initiated a First International T-cell Workshop, an effort involving the exchange of a range of data and the sharing of results. This report describes the characterization of recombinant preparations of GAD65, proinsulin, and IA-2 and their use in detecting autoreactive T-cells in type 1 diabetes.

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EU, endotoxin unit; PBMC, peripheral blood mononuclear cell; PI, proinsulin; TCL, T-cell line; TCC, T-cell clone; TT, tetanus toxoid.

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candidate autoantigen preparations between laboratories. Several important findings were reported after this effort (13), and, in particular, the workshop highlighted the critical importance of carefully evaluating the quality of islet autoantigen preparations before using them. Some of the antigen preparations used in the First International T-Cell Workshop inhibited TCC proliferation to an unrelated antigen or were not recognized by TCCs known to be specific for the index autoantigen. Clearly, these preparations would be unlikely to perform optimally in eliciting spontaneous T-cell autoreactivity in peripheral blood.

In this study, we sought to evaluate the quality of available preparations of three major candidate islet autoantigens, namely GAD65, proinsulin (PI), and the protein tyrosine phosphatase–like molecule IA-2. Quality was assessed by the measurement of endotoxin levels, the ability of preparations to stimulate proliferation of a panel of known antigen-specific TCCs or T-cell lines (TCLs), and their effect on peripheral blood T-cell proliferation to an unrelated antigen. The goal of this study was to identify and standardize antigen preparations for wider distribution, to serve as the standard in research programs designed to evaluate technologies for the detection of autoreactive T-cells.

**RESEARCH DESIGN AND METHODS**

**Antigens.** The characteristics of the antigen preparations assessed are shown in Table 1. GAD65 was prepared in insect cells using the baculovirus expression system in two different centers. GAD65 was also generated in *Escherichia coli* using a modification of previously described techniques (14) and including a 14 kDa NH2-terminal biotinylation tag. *E. coli* GAD65 was immunoprecipitated by sera from type 1 diabetic patients. A chimeric GAD molecule, comprising residues 1–151 of GAD67 and residues 96–285 of GAD65, was expressed in yeast cells (GAD65/67yeast). GAD65/67yeast is enzymatically active and reactive in immunoprecipitation assays and by enzyme-linked immunosorbent assay with diabetic sera (15). The intracellular portion of IA-2 was available expressed in baculovirus in two preparations and also in *E. coli*. PI was expressed in *E. coli* and made available by Eli Lilly. Two preparations of insulin were available, expressed in yeast cells. All preparations had purities >90%, as assessed by SDS-PAGE. Endotoxin levels were determined by the Limulus lysate assay.

**Proliferation of TCCs and TCLs to candidate antigens.** Several TCLs and TCCs specific for the islet autoantigens were available for testing the quality of the preparations (Table 2). To examine potential toxic effects of antigens or their buffer constituents, standard peripheral blood T-cell proliferation assays were carried out with a single donor using tetanus toxoid (TT) (Pasteur Merieux, Maidenhead, U.K.) as stimulating antigen, as follows. PBMCs were prepared by density gradient centrifugation and seeded into 96-well U-bottom tissue culture plates at a density of 150,000 per well in 125 μl RPMI-1640 (Life Technologies, Paisley, U.K.)/10% human AB serum (PAA Laboratories, Middlesex, U.K.) containing TT (final assay concentration 50 μg/ml). For testing antigen toxicity, a further 25 μl of medium was added, supplemented with workshop antigens at doubling dilutions in RPMI-1640/10% AB serum to achieve final assay concentrations of 0.156–20 μg/ml. For testing buffer toxicity testing.

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**TABLE 2**

<table>
<thead>
<tr>
<th>Islet antigen</th>
<th>Designation</th>
<th>Epitope</th>
<th>HLA restriction element</th>
<th>Provider</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD65</td>
<td>TCL 6/4</td>
<td>270–283</td>
<td>DRB1*0401</td>
<td>Dr. Josef Endl, Roche Diagnostics, Germany</td>
<td>7</td>
</tr>
<tr>
<td>GAD65</td>
<td>P-TCC</td>
<td>248–257</td>
<td>DRB5*0101</td>
<td>Dr. Peter Van Endert, Paris, France</td>
<td>8</td>
</tr>
<tr>
<td>GAD65</td>
<td>P-TCL</td>
<td>556–575</td>
<td>DRB1<em>1501/DRB5</em>0101</td>
<td>Dr. Peter Van Endert</td>
<td>8</td>
</tr>
<tr>
<td>GAD65</td>
<td>PMI#11</td>
<td>339–352</td>
<td>DR3</td>
<td>Dr. Bart Roep, Leiden, the Netherlands</td>
<td>16</td>
</tr>
<tr>
<td>IA-2</td>
<td>TCL 9655</td>
<td>751–770</td>
<td>DR4</td>
<td>Dr. Bart Roep</td>
<td>10</td>
</tr>
<tr>
<td>IA-2</td>
<td>TCR 9665</td>
<td>831–860</td>
<td>DBP1*0401</td>
<td>Dr. Bart Roep</td>
<td>10</td>
</tr>
<tr>
<td>Insulin</td>
<td>INS-1</td>
<td>B-chain 11–27</td>
<td>DRB1*1601</td>
<td>Dr. Bart Roep</td>
<td>9</td>
</tr>
</tbody>
</table>

*Pyridoxal phosphate; †2-aminooxytosylsulphonylurea bromide; §phenylmethylsulphonylfluoride; £EU/ml at antigen concentration of 10 μg/ml.

**TABLE 1**

<table>
<thead>
<tr>
<th>Islet antigen</th>
<th>Designation</th>
<th>Expression system</th>
<th>Buffer constituents</th>
<th>Stock concentration (mg/ml)</th>
<th>Endotoxin level (EU/ml§)</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD65</td>
<td>GAD65baculo-A</td>
<td>Baculovirus</td>
<td>N/A</td>
<td>1.9</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>GAD65</td>
<td>GAD65baculo-B</td>
<td>Baculovirus</td>
<td>50 mmol/l hepes, 1 mmol/l PLP*, 10% glycerol</td>
<td>0.3</td>
<td>&lt;0.6</td>
<td>8</td>
</tr>
<tr>
<td>GAD65</td>
<td>GAD65Ecoli</td>
<td>E. coli</td>
<td>4 mmol/l hepes, 50 μmol/l PLP*, 2.5 mmol/l glutathione</td>
<td>1.5</td>
<td>&lt;0.6</td>
<td>8</td>
</tr>
<tr>
<td>GAD65/67</td>
<td>GAD65/67yeast</td>
<td>Yeast</td>
<td>50 mmol/l K2HPO4, 1mmol/l sodium glutamate, 20 mmol/l PLP, 1mmol/l 2-AET†, 1mmol/l PMSF‡, 300mmol/l KCl, 30% glycerol</td>
<td>0.35</td>
<td>0.9</td>
<td>15</td>
</tr>
<tr>
<td>IA-2</td>
<td>IA-2baculo-A</td>
<td>Baculovirus</td>
<td>200 mmol/l NaCl, 10% glycerol, 25 mmol/l</td>
<td>0.4</td>
<td>&lt;0.6</td>
<td>8</td>
</tr>
<tr>
<td>IA-2</td>
<td>IA-2baculo-B</td>
<td>Baculovirus</td>
<td>Phosphate buffered saline, pH 7.4</td>
<td>0.3</td>
<td>ND</td>
<td>8</td>
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<tr>
<td>IA-2</td>
<td>IA-2Ecoli</td>
<td>E. coli</td>
<td>Phosphate buffered saline</td>
<td>0.5</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>Proinsulin</td>
<td>PInEcoli</td>
<td>E. coli</td>
<td>Phosphate buffered saline</td>
<td>5.0</td>
<td>&lt;0.6</td>
<td>8</td>
</tr>
<tr>
<td>Insulin</td>
<td>InsNovo</td>
<td>Yeast</td>
<td>Phosphate buffered saline</td>
<td>5.0</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>Insulin</td>
<td>InsSigema</td>
<td>Yeast</td>
<td>Phosphate buffered saline</td>
<td>2.0</td>
<td>ND</td>
<td>8</td>
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</tbody>
</table>
constituent toxicity, solutions were prepared in 100 mmol/l Na2HPO4, pH 7.4, and sterile filtered. Stock buffers were prepared based on the constituents known to be present in antigen preparations (Table 1) or reagents used in antigen purification, as follows: 100 mmol/l Na2HPO4, 100 mmol/l Tris, 2.5 mmol/l reduced glutathione, 1 mmol/l phenylmethylsulfonylfluoride, 300 mmol/l KCl, 1 mmol/l glutamic acid, 30% glycerol, 2 mmol/l EDTA, 0.01% Triton X-100, and 50 µmol/l pyridoxal phosphate (all from Sigma Chemicals, Poole, U.K.). PBMCs were seeded as described above in medium containing TT, and a further 25 µl was added, either as a pure test buffer or as a series of seven doubling dilutions in RPMI-1640/10% AB serum. Plates were incubated for 6 days at 37°C in 5% CO2 and pulsed with 0.5 µCi per well [3H]-thymidine for the last 18 h before harvesting onto glass fiber filters and direct plate counting in a Matrix 9600 counter (Packard Instruments, Pangbourne, U.K.). All assays (antigen and buffer toxicity) were performed in triplicate on the same day, and data presented are representative of three separate experiments on the same donor.

RESULTS

TCC responses to GAD65 preparations. A range of TCCs and TCLs raised in different laboratories against different preparations of GAD65 and reacting with different regions of the molecule were available for study. TCL 6/4 (7), raised against recombinant human GAD65baculo–A expressed in baculovirus, was stimulated in a dose-dependent manner by an equivalent but different preparation of GAD65 expressed in baculovirus (GAD65baculo–B) (Fig. 1A). TCL 6/4 was also stimulated with a similar dose dependency by GAD65/67yeast1 fusion protein, and no response was seen to GAD65Ecoli at any concentration. P-TCC, a TCC raised against baculovirus-derived GAD65 of a different source (8), was stimulated to proliferate by the same antigen prepared for the workshop (GAD65baculo–B). A similar profile of proliferation was seen to the GAD65/67yeast1 fusion protein, with evidence of inhibitory effects of this preparation at concentrations >1 µg/ml (Fig. 1B). P-TCC also proliferated in response to the GAD65Ecoli preparation, but with a shallower dose-response relationship than that observed with the baculovirus preparations. A similar profile was seen for P-TCL also raised against baculovirus-derived GAD65 (8), which proliferated in response to all three GAD65 preparations (Fig. 1C). The TCC designated PM1#11 (16) was raised against human baculovirus-derived GAD65 of a different source (Dr. T. Dyrberg, Novo Nordisk) and showed optimal proliferation to both GAD65 preparations generated using baculovirus. Proliferation was also seen to GAD65/67yeast1 fusion protein, whereas there was no evidence of proliferation to GAD65Ecoli (Fig. 1D). In quality control assays for the First International T-Cell Workshop, this clone responded to low concentrations of E. coli–expressed GAD65, with a declining dose-response after 1 µg/ml of this antigen (13).

TCL responses to IA-2. TCLs (9656 and 9665) raised against IA-2 expressed in E. coli (10) were tested for proliferation against a different E. coli preparation and two IA-2 preparations expressed in baculovirus (Fig. 2). TCL 9656 was stimulated efficiently by the IA-2baculo–A preparation, the IA-2baculo–B preparation, and the IA-2 expressed in E. coli. Although TCL 9655 responded to all three preparations, it was most efficiently stimulated by IA-2baculo. TCL 9665 reacts to peptides in region 751–770 of IA-2, a region known to be naturally processed and presented by HLA-DR4 (17). One possible explanation for the differential pattern of responsiveness to different preparations seen in TCL 9665 is that although there are IA-2 peptide-specific T-cells present, the line also contains cells reactive with bacterial contaminants contained in the E. coli preparation used to generate the line.

TCC responses to PI. No TCLs or clones specifically raised against PI were available for use in evaluating the PI responses to GAD65.
preparation. However, we were able to evaluate the efficiency with which an epitope within the insulin B-chain was presented from the intact PI molecule using a TCC raised against insulin (9). This TCC recognizes residues 11–27 in the insulin B-chain and was stimulated by two different preparations of insulin (Fig. 3). In contrast, the PI preparation stimulated this clone only weakly.

Effect of antigen preparations and buffer constituents on T-cell proliferation to TT. To examine whether antigen preparations were intrinsically inhibitory or stimulatory, conventional T-cell proliferation assays were spiked with various amounts of antigen, starting at a final concentration of 20 μg/ml, with seven doubling dilutions down to 156.25 ng/ml to give a total of eight determinations for each antigen. The baculovirus-expressed preparations of GAD65 (P-GAD65bacule) and IA-2 (P–IA-2 bacule), and the preparation of PI showed little effect on TT-induced proliferation (Fig. 4). In contrast, the yeast-cell preparation of GAD65/67yeast inhibited TT-induced proliferation at the highest antigen concentration. GAD65 Ecoli consistently and markedly inhibited TT-induced proliferation at all concentrations.

To examine which buffer constituents may contribute to inhibition of T-cell proliferation, experiments with a similar design were carried out in which ≤25 μl of the TT proliferation assay medium was replaced with single buffers. The results indicate that Na₂HPO₄, Tris, phenylmethylsulfonyl fluoride, glutathione (Fig. 5A), glutamic acid, and pyridoxal phosphate (Fig. 5B) are all well tolerated. In contrast, KCl, glycerol, Triton X-100, and EDTA showed marked inhibitory effects at high concentrations.

DISCUSSION
In the First International Workshop for Standardization of T-cell Assays in Type 1 Diabetes, numerous islet autoantigen preparations were assessed for their ability to stimulate peripheral blood T-cells (13). The main conclusion drawn from that workshop was that the major factors affecting reliable and consistent detection of islet autoreactive T-cells were the limitations of current assay technology and the quality of antigen preparations. Because the development of assay technology is in turn heavily

FIG. 3. Proliferative responses of TCC INS1 to PI (PIEcoli) (●) and two different preparations of insulin, one from Novo Nordisk (□) and one from Sigma Chemical (▲), expressed in yeast. For details of clones, see TABLE 2. For details of antigens, see Table 1.

FIG. 4. Proliferative responses of PBMCs from a healthy donor to TT (TT) in the absence or presence of increasing concentrations of different antigen preparations, to detect inhibitory or stimulatory effects. Antigen preparations tested were GAD65bacule-B (●), GAD65Ecoli (▲), GAD65/67yeast (□), IA-2bacule-A (▲), and PIEcoli (●). Means and SDs of the response to TT are shown by the small circle and error bars. For details of antigens, see Table 1.

FIG. 5. Proliferative responses of PBMCs from a healthy donor to TT (TT) in the absence or presence of increasing volumes of different antigen preparations, to detect inhibitory or stimulatory effects. The following buffers were tested in A: 100 mmol/l Na₂HPO₄ (●), 100 mmol/l Tris (▲), 2.5 mmol/l reduced glutathione (▲), 1 mmol/l phenylmethylsulfonylfluoride (□), and 300 mmol/l KCl (□). In B buffers tested were: 1 mmol/l glutamic acid (●), 30% glycerol (■), 2 mmol/l EDTA (△), 0.01% Triton X-100 (●), and 50 μmol/l pyridoxal phosphate (□). Mean and SDs of the response to TT are shown by the small circle and error bars.
reliant on antigen quality, the strategy adopted for subsequent workshops has been to address each factor in an iterative step-wise program. The first component of that process forms the basis for the present study, in which we acquired and evaluated the quality of several preparations of the major islet autoantigens GAD65, IA-2, and PI. Assessment of quality in our study has made use of an exceptional resource, namely the availability of a panel of islet antigen–specific TCCs and TCLs.

Our results extend preliminary findings made in the First International T-Cell Workshop, demonstrating that preparations of GAD65 expressed using baculovirus are of superior quality compared with preparations of GAD65 obtained from \textit{E. coli}. This conclusion is based on two pieces of evidence. First, several baculovirus GAD65 preparations were able to stimulate GAD65-specific TCCs that had been raised independently in different laboratories using different GAD65 preparations. This is an important finding because in the First International T-Cell Workshop, some TCLs specific for GAD65 were only responsive to the antigen preparation against which they were generated. Second, as a further demonstration of their quality, tested baculovirus GAD65 preparations did not inhibit spontaneous peripheral blood T-cell proliferation to an unrelated antigen (TT) at typical working concentrations. It is reasonable to conclude from these results that GAD65 preparations expressed in baculovirus can be used to examine peripheral blood T-cell reactivity.

A GAD65/67 chimeric molecule expressed in yeast was also able to induce GAD65-specific TCCs to proliferate efficiently. The GAD65/67 chimera is recognized by sera containing conformation-dependent GAD65 autoantibodies and is also enzymatically active (15). However, this GAD65/67 chimera displayed moderate toxicity at working concentrations in the T-cell inhibition assay. It is not clear whether the toxicity relates to buffer constituents, some of which were also toxic, or to unidentified yeast-cell contaminants. A further limitation of this construct is the absence of the first 95 amino acids of GAD65, which may contain T-cell epitopes.

In the First International T-Cell Workshop, GAD65 expressed in \textit{E. coli} completely inhibited proliferation of an islet antigen–specific TCC and proliferation of PBMCs to a third-party antigen in co-culture experiments. In contrast, the \textit{E. coli} preparation used in the current study stimulated two of four GAD65-specific TCCs. This antigen preparation was also the most toxic in inhibiting proliferation to TT. Buffer constituents are the most likely cause of these toxic effects because endotoxin levels in these preparations were low (all <0.9 endotoxin units [EU] per 10 \(\mu\)g protein). This is a considerable improvement compared with the endotoxin contamination of several of the preparations of the First International T-cell Workshop (\textit{E. coli} expressed in GAD65 6.0 EU per 50 \(\mu\)g; IA-2 expressed in \textit{E. coli} 30–60 EU per 50 \(\mu\)g).

Two TCLs reactive with IA-2 were available for study. Broadly similar levels of reactivity were seen with TCLs 9655 and 9665 in response to the different preparations. The high level of stimulation of TCL 9665 to IA-2\textsubscript{Ecoli} may be attributable to the presence of T-cells within the line that respond to bacterial contaminants, such as heat shock proteins. In addition, the IA-2\textsubscript{baculo}–A preparation was shown to not inhibit T-cell responses to an unrelated antigen (TT).

Only a single PI preparation and only a single potentially reactive TCC were available for evaluation in this workshop. In a study in which this antigen preparation was used to detect peripheral blood reactivity to PI, spontaneous proliferation was rarely detected and was found at a similar frequency in patients and control subjects (18). Although the PI preparation showed low levels of toxicity, it was unable to elicit proliferation from an insulin B-chain–specific TCC. It would appear that the relevant B-chain epitope is not processed and presented by the HLA DR allele (HLA-DRB1*1,601) through which the clone is restricted. If this proves to be a more universal finding for other alleles, it would restrict the utility of this preparation in assay development. This unexpected finding will require further elucidation using additional PI or insulin-specific TCCs as they become available.

The results of the stimulation of GAD65– and IA-2–specific clones by different preparations of GAD65 and IA-2 are of interest because they illustrate the importance and feasibility of defining standard preparations and making these available. It is clear from our data that although different islet antigen-specific clones may have slightly different response profiles, possibly caused by different kinetics of proliferation for individual clone/antigen combinations, these clones do respond to the different antigen preparations. Translating these differing effects of antigen into assays performed using polyclonal populations of T-cells from peripheral blood to detect autoreactivity, it is clear that the use of different preparations, even those made in similar expression systems, may provide different results. This stresses the importance of deriving a set of workshop standards and highlights the value of the panel of islet autoreactive TCCs we have used.

The inhibition assays indicated that some of the observed effects involving antigen preparations are attributable to buffer constituents. These experiments were designed to reproduce the most likely final buffer concentrations used when stock antigens at low concentrations (e.g., 0.2–0.3 mg/ml) are used at final concentrations as \(\pm 50 \mu\)g/ml. According to our results, it is clear that PMSF, Tris, glutathione, potassium chloride, glycerol, pyridoxal phosphate, Triton X-100, and EDTA must be used with caution in antigen preparations. Finally, it is worth noting that tests such as these, using TCCs and TCLs that are largely or entirely specific for a given autoantigen, cannot reveal whether antigen preparations will stimulate T-cells in a non–antigen-specific manner and to what degree. It will be important in future evaluations of antigen preparations to include panels of healthy nondiabetic donors for evaluating such nonspecific effects.

In summary, we have evaluated several recombinant preparations of the major autoantigens GAD65, IA-2, and PI. Our data indicate profound effects of different preparations on cloned T-cell responses and on peripheral blood responses to unrelated antigens. Most of the unwanted effects are likely to be attributable to contaminants or toxic buffer constituents. Our analysis identifies preparations of GAD65, IA-2, and PI that have favorable characteristics for evaluation in assay formats for the detection
of autoreactive T-cells, and these are now available for testing in individual laboratories.

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REFERENCES