Improved T cell assay for identification of type 1 diabetes patients

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ABSTRACT

Diabetes mellitus is comprised primarily of two clinically separate diseases: type 1 (T1D) and type 2 diabetes (T2D). T1D is a cell-mediated autoimmune disease directed against the beta cells and characterized by autoantibody (Ab) and T cell reactivity to islet proteins whereas, T2D is non-autoimmune. Despite the fact that the pathological process in autoimmune diabetes involves T cells, immune markers of diabetes have primarily centered on the presence of circulating serum islet autoantibodies. In two masked NIH sponsored workshops, our cellular immunoblotting T cell assay, which uses isolated human islets separated into 18 molecular weight fractions, has been validated to be able to distinguish T1D patients from controls with excellent specificity and sensitivity. In this study, we utilized the first workshop to select eight molecular weight fractions of human islets that were the most discriminatory between T1D patients and controls. Using these eight molecular weight fractions identified in the first workshop, we validated the preferential recognition of these 8 blot sections in a second workshop. We then re-calculated the sensitivity and specificity of the cellular immunoblotting assay for both workshops using only the data from these 8 blot sections. We observed increases in both sensitivity and specificity compared to the original workshop data for both workshops. The use of 8 instead of 18 molecular weight regions allows for a significant reduction in the amount of blood needed from patients, thus allowing cellular immunoblotting to be performed on pediatric patients participating in immunomodulatory studies. This improved T cell assay, which directly measures islet reactive T cell responses in autoimmune diabetes patients with excellent sensitivity and specificity, will likely improve patient follow-up during intervention studies.

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1. Introduction

Autoimmune diseases affect approximately 5–7% of the adult population in North America and Europe (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997) and include type 1 diabetes (T1D). T1D is characterized by the presence of autoantibodies (Lernmark, 1987; Palmer et al., 1983), insulitis (Gepts, 1965) and selective immune-mediated destruction of pancreatic beta cells (Gepts, 1965; Rahier et al., 1983). The pathogenesis of T1D is believed to be cell-mediated since T cells but not antibodies are necessary to transfer disease in animal models and human T1D (Bendelac et al., 1987; Miller et al., 1988; Lampeter et al., 1993). In fact, bone marrow cells have been demonstrated to transfer T1D between HLA-identical siblings (Lampeter et al., 1993). In contrast, type 2 diabetes (T2D) is believed not to be initiated by an autoimmune attack on the Beta cells. However, a subgroup of phenotypic T2D patients with autoantibodies and T cells responsive to islet cell proteins similar to T1D patients (type 1.5 diabetes/LADA), have been identified previously by us and many other groups (Tuomi et al., 1993; Lohmann et al., 1997; McCance et al., 1997; Brooks-Worrell et al., 1999; Juneja et al., 2001). Thus, T
cells may play a role in the pathogenesis of diabetes in patients with phenotypic T1D and T2D.

Islet proteins currently utilized in most T cell assays were identified based on autoantibody and not T cell reactivity. Since autoantibodies and T cells don’t necessarily recognize similar proteins or epitopes, many important T cell stimulatory antigens may have been missed. This may be an important component in the low specificity and sensitivity for T1D of the many T cell assays currently employed to study the diabetes disease process. Recently, results of a TrialNet workshop (Herold et al., 2007) validated earlier results of an Immune Tolerance Network (ITN) T cell workshop (Seyfert-Margolis et al., 2006) demonstrating that our cellular immunoblotting assay could distinguish T1D patients from controls with excellent specificity and sensitivity. In this study, we have moved one step closer toward identification of islet proteins stimulatory to T cells from T1D patients and have developed a T cell assay which can be more easily used to follow patients in intervention studies. We have identified molecular weight regions that contain islet proteins stimulatory to T cells from T1D patients in an ITN sponsored T cell workshop, selected the most discriminatory regions, and subsequently verified the results in a second T cell workshop sponsored by TrialNet. Thus, we have developed a T cell assay with improved specificity and sensitivity. This will likely lead to improved monitoring of T1D patients participating in immunomodulation studies.

2. Methods

2.1. Subjects

In the ITN workshop (Seyfert-Margolis et al., 2006), masked blood samples were obtained from 33 normal controls and 17 recently diagnosed young T1D patients and shipped overnight, without freezing. In the TrialNet workshop (Herold et al., 2007), masked blood samples were obtained from 74 normal controls and 48 recently diagnosed young T1D patients and shipped overnight, without freezing. Blood samples were processed and cells cultured within 24 h of blood collection.

2.2. Cellular immunoblotting

Cellular immunoblotting was performed as previously described (Brooks-Worrell et al., 1996). Briefly, isolated and purified human islets were obtained from the NIH Islet Consortium. Islet cells were subjected to preparative 10% SDS–PAGE. Following electrophoresis, the gels were electroblotted onto nitrocellulose (BioRad, Richmond, CA) at 30 mA overnight, nitrocellulose particles prepared, and the nitrocellulose particles used to stimulate PBMCs in vitro.

A stimulation index (SI) for each molecular weight section was calculated as follows:

\[
\text{Stimulation Index (SI)} = \frac{\text{Mean CPM experimental wells} - \text{Mean CPM control wells}}{\text{Mean CPM control wells}}
\]

Control wells contained nitrocellulose particles without antigen and cells only. Positive proliferation was considered to be an SI > 2.0 which corresponds to greater than the mean + 3 SD of control values (Brooks-Worrell et al., 1996). A designation of T1D was given to samples where 4–18 blot sections demonstrated a positive SI (> 2.0). Antigen doses, consistency among various islet antigen preparations, and specificity of PBMC responses of T1D patients to the islet protein preparations have been previously described (Brooks-Worrell et al., 1996). PBMC responses to tetanus toxoid were used as a control antigen along with PBMC responses to mitogens. An adequate number and quality of human islets has been available through the NIH Islet Consortium. Islet antigen preparations used in each of the workshops are prepared from islets obtained from a single donor. New islet antigen preparations are analyzed alongside of previously established islet preparations to insure quality control and consistency of results among the islet antigen preparations.

3. Results

Using our cellular immunoblotting results from the ITN T cell workshop (Seyfert-Margolis et al., 2006), we established a cut-off for preferential recognition by T cells to the molecular weight regions. We set the cut-off for preferential

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**Table 1**

Molecular weights of identified molecular weight regions of interest from the ITN workshop (Seyfert-Margolis et al., 2006).

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Blot sections</th>
<th>Molecular weight (kD)</th>
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<tbody>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>126–145</td>
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<td></td>
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<tr>
<td></td>
<td>9</td>
<td>10–14</td>
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recognition to be a positive value of >40% of T1D patients and <15% of controls. Fig. 1 illustrates the molecular weight regions of interest from the ITN workshop data. The molecular weights of the preferentially recognized regions of interest identified from the ITN workshop are shown in Table 1. Even though the highest molecular weight region (>200 kD) demonstrated recognition by T cells in both workshops, we decided not to use this blot section based on the concern for protein complexes and cellular debris present at the top of gels.

We then utilized the identified eight molecular weight fractions selected from the ITN T cell workshop, and validated the preferential recognition of these 8 blot sections using a second workshop, Trialnet T cell workshop. Using the newly identified preferentially recognized 8 molecular weight regions, we re-calculated the sensitivity and specificity of the cellular immunoblotting assay and compared these specificities and sensitivities calculated from the original ITN and TrialNet workshop data which were based on the 18 molecular weight regions (Table 2). We observed an increase from the previously reported sensitivity of 94% and specificity of 83% to an improved sensitivity of 100% and a specificity of 96% for the ITN workshop data. For the TrialNet data using 18 molecular weight regions, the original sensitivity was reported to be 74% with a specificity of 88%. The revised specificity and sensitivity using the identified preferentially recognized 8 blots were 97% and 94% respectively (Table 2).

We next analyzed the results of the TrialNet workshop to identify which of the molecular weight fractions would also be considered preferentially recognized if the same criteria (T1DMs >40% and controls <15%) were applied. Fig. 2 and Table 3 demonstrates the preferentially recognized molecular weight regions according to the Trialnet data. Interestingly, the molecular weights of the T cell stimulatory islet proteins identified from the ITN workshop were similar to the molecular weights of the T cell stimulatory islet proteins preferentially recognized by T cells from type 1 diabetes patients in the TrialNet workshop (Tables 1 and 3).

### 4. Discussion

Pancreatic beta cells are destroyed by T cells during the T1D disease process. Therefore, immunomodulatory trials designed to modulate or inhibit the autoimmune T cell destruction of beta cells during or after the development of clinical T1D are in desperate need of T cell assays for monitoring the success and progression of these trials. Previously, monitoring has been performed by assessment of C-peptide and/or autoantibody responses. However, approximately 19% of patients newly diagnosed clinically as autoimmune T1D have been demonstrated to be autoantibody-negative at the time of diagnosis (Wang et al., 2007).

We have been investigating T cell responses in classic T1D and in adult phenotypic T2D patients and have identified T cell responses to islet proteins (Brooks-Worreul et al., 1996, 1999, 2001, 2004). Moreover, we (Goel et al., 2007) have also demonstrated a correlation between T cell positivity and diminished beta cell function. Our data demonstrate that identifying patients with diabetes who have T cell responses to islet proteins identifies the patients with a more severe beta cell lesion compared with autoantibody assessment alone. Therefore, the use of T cell assays to measure T cell responses to islet proteins in diabetes patients has significant clinical relevance.

Cellular immunoblotting is a technique originally developed by Abou-Zeid et al. (1987) and subsequently utilized by many investigators working in various model systems to identify proteins directly stimulatory to T cells (Young and Lamb, 1986; Lee et al., 1989; Melby and Sacks, 1989; Pham et al., 1989; Brooks-Worrell and Splitter, 1992; Freeman and Weetman, 1992; VanNoort et al., 1993). We have adapted

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Table 2

<table>
<thead>
<tr>
<th>T cell workshops</th>
<th>Analysis using original 18 blots</th>
<th>Analysis using new 8 blots</th>
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<tr>
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<td>ITN*</td>
<td>TrialNet**</td>
</tr>
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<td>ITN workshop</td>
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<tr>
<td></td>
<td>Sensitivity</td>
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<td></td>
<td>94%</td>
<td>74%</td>
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<tr>
<td></td>
<td>Specificity</td>
<td>100%</td>
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Table 3

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<tr>
<th>Analysis</th>
<th>Blot sections</th>
<th>Molecular weight (kD)</th>
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</thead>
<tbody>
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<td>TrialNet workshop</td>
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<tr>
<td>5</td>
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<tr>
<td>17</td>
<td>13</td>
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</table>

Fig. 2. Preferentially recognized molecular weight regions of islet proteins identified using PBMCs from T1D patients and controls participating in the TrialNet workshop (Herold et al., 2007). Criteria for preferential recognition were positive responses >40% of T1D patients and <15% of controls. Preferential molecular weight regions are identified by an arrow.
cellular immunoblotting to follow T cell responses to islet proteins in diabetes patients. Unfortunately, incorporation of 18 molecular weight regions demands a relatively large volume of blood (30 cc) thus limiting the assay’s use in pediatric populations participating in immunomodulatory trials. In this study, we were able to identify 8 molecular weight regions, preferentially recognized by T cells from T1D patients participating in the ITN workshop and validated the discriminatory ability of the selected blot sections using a second T cell workshop, sponsored by Trialnet. Reassessing the sensitivity and specificity of the cellular immunoblotting assay utilizing only the preferentially recognized molecular weight regions (selected from the ITN T cell workshop), increased the specificity and sensitivity of the cellular immunoblotting assay in both workshops. Moreover, even though the islet preparations used in both workshops were produced from islets from different donors, the molecular weight regions preferentially recognized were similar. Three of the molecular weight regions in the TrialNet workshop (blots 3, 9, 11) with molecular weights of 131–151 kD, 48–56 kD, and 34–41 kD did not reach the cut-off of >40% of T1D patients responding but the responses were close to the cut-off with 38% of the T1D patients responding and <15% of normal controls responding. However, similar molecular weight regions, blot 3 with molecular weight of 126–145 kD, blot 9 with molecular weight 43–53 kD and blot 10 molecular weight 36–43 kD were recognized in the ITN workshop (>40% T1DM and <15% controls) and thus included in the calculation of the sensitivity and specificity. The highest molecular weight region (>200 kD) was not included in the identified 8 chosen regions due to the possibility of this region containing protein complexes and cellular debris. The eventual goal will be to identify proteins that could be used in T cell assays for immunomonitoring of studies related to autoimmune diabetes. Until the individual islet proteins stimulatory to the T cells in each molecular weight region are identified, we will continue to focus on molecular weight regions. Future studies will hopefully allow us to identify and isolate the individual islet proteins.

Decreasing the number of molecular weight regions utilized by half would allow for 1/2 the amount of blood to be drawn, thus permitting the use of cellular immunoblotting in pediatric immunomodulatory trials. Furthermore, our report points to molecular weight regions where individual islet proteins stimulatory to T cells from autoimmune diabetes patients may be isolated in future studies. Even though the specific islet proteins stimulatory to T cells from T1D patients in our assay are not known at this time, the identification of similar molecular weight regions in two separate workshops years apart using islet antigen preparations produced from separate donors is remarkable. Identification of specific stimulatory islet proteins should contribute significantly to our understanding of the T1D disease process as well as the development of potential antigen-based intervention strategies.

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References


