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Research paper

Improved T cell assay for identification of type 1 diabetes patients

Barbara Brooks-Worrell *, Adelaide Warsen, Jerry P. Palmer

Veterans Affairs Puget Sound Health Care System, Department of Medicine, Division of Metabolism, Endocrinology, and Nutrition, University of Washington, Seattle, Washington, United States

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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

Abbreviations: HLA, human leukocyte antigen; CPM, counts per minute; PMBCs, peripheral blood mononuclear cells; ITN, immune tolerance network; T1D, type 1 diabetes mellitus; T2D, type 2 diabetes mellitus; Ab, autoantibodies; NIH, National Institutes of Health; LADA, latent autoimmune diabetes of adults.

* Corresponding author. Veterans Affairs Puget Sound Health Care System, 1660 S. Columbian Way, Building 1, Room 609, Seattle, WA 98108, United States. Tel.: +1 206 764 2696; fax: +1 206 764 2615.
E-mail address: bbrooks@u.washington.edu (B. Brooks-Worrell).

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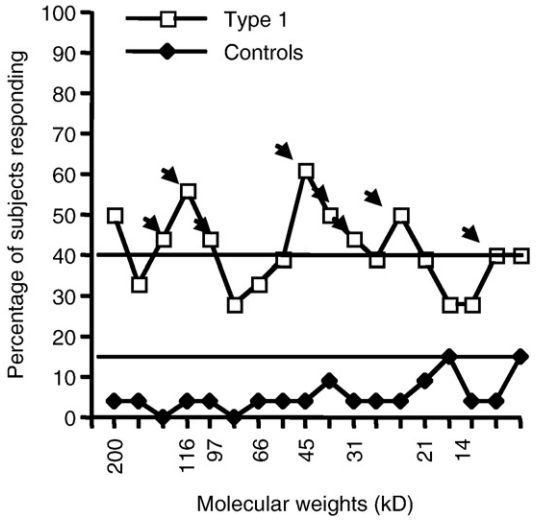

Fig. 1. Preferentially recognized molecular weight regions of islet proteins identified using PBMCs from T1D patients and controls participating in the ITN workshop (Seyfert-Margolis et al., 2006). Criteria for preferential recognition were positive responses >40% of T1D patients and <15% of controls. Preferential molecular weight regions are identified by an arrow.

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 a 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:

$$\text{Stimulation Index (SI)} = \frac{\text{Mean CPM experimental wells}}{\text{Mean CPM control wells}}.$$

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

Table 1
Molecular weights of identified molecular weight regions of interest from the ITN workshop (Seyfert-Margolis et al., 2006).

Analysis	Blot sections	Molecular weight (kD)
ITN workshop	1	182–200
	3	126–145
	4	104–126
	5	88–104
	9	43–53
	10	36–43
	11	30–36
	13	21–25
	17	10–14

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