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Research paper

Triple chimeric islet autoantigen IA2–ZnT8WR to facilitate islet autoantibody determination

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ABSTRACT

Type 1A diabetes is strongly associated with the presence of islet autoantibodies. Large scale population screening of islet autoantibodies is essential for many different national and international studies related to defining subtypes of diabetes, the natural history of the disease, and for trials of prevention. Testing for relevant autoantibodies has become more difficult as the number of important autoantibodies/epitopes increases. In the present study, we created a chimeric protein, IA2–ZnT8WR, with two major islet autoantigens, IA-2 and the recent Zinc transporter 8 (ZnT8). The chimeric molecule included both common polymorphisms of the ZnT8 molecule, arginine or tryptophan at position 325. Serum samples from 284 patients with newly diagnosed diabetes, 10 prediabetics, and 110 age-matched normal controls were analyzed for islet autoantibodies reacting with the IA2–ZnT8WR molecule. Autoantibodies to the chimeric molecule were compared to reactivity with individual assays detecting autoantibodies reacting with the separate molecules (IA-2, ZnT8-R and ZnT8-W). With this chimeric protein antigen, IA2–ZnT8WR, one radioassay is able to detect autoantibodies to IA-2 and to both major forms of ZnT8 (100% sensitivity, 100% unchanged specificity, relative to individual molecules). The chimeric assay provides an efficient and economical technique to screen for islet autoantibodies reacting with IA-2 and ZnT8.

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1. Introduction

For more than a decade, multiple "biochemically-defined" anti-islet autoantibodies have been recognized as important predictive markers of developing type 1A diabetes (Verge et al., 1996; Bingley et al., 1997; Greenbaum et al., 1999). The autoantibodies are characteristically detected by most investigators with fluid phase radioassays to enhance specificity utilizing *in vitro*-translated antigens. Autoantibodies reacting with insulin, glutamic acid decarboxylase (GAD65), and the protein tyrosine phosphatase (IA-2) have been described, and sensitive autoantibody radioassays have been developed. These autoantibodies can be present years before diabetes onset, and a high risk of progression to diabetes (>90%) is associated with the presence of two or more of these autoantibodies. Multiple prospective studies of both relatives of patients with type 1 diabetes and the general population have demonstrated the predictive potential of these autoantibody assays.

Recently, Wenzlau et al. (2007) identified a new islet autoantigen, Zinc transporter 8 (ZnT8). The radioassay for autoantibodies to ZnT8 (ZnT8Ab) was established and confirmed in the 2007 and 2009 DASP (Diabetes Autoantibody Standardization Program) workshops. ZnT8Ab were detected in 63% of new-onset diabetic patients and increased overall autoantibody positivity by 5% among new-onset patients who were negative for the previously defined autoantibodies (mIAA, GAD65Ab, IA-2Ab). Studies in prediabetic patients

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demonstrated that ZnT8Ab predicts progression to diabetes and enhances the current prediction of diabetes (Wenzlau et al., 2008a).

In the current study, we constructed a chimeric protein with the intracytoplasmic domain of IA-2 (IA-2ic) and two polymorphic variants of ZnT8 (Wenzlau et al., 2008b). Utilizing this chimeric protein, with one assay, we are able to detect autoantibodies to both IA-2 and ZnT8 polymorphic variants which should provide an efficient tool for autoantibody screening of large populations.

2. Materials and methods

2.1. Subjects

Serum samples from a total of 284 with 2 sets of newly diagnosed diabetic patients at the Barbara Davis Center for Childhood Diabetes were analyzed in the present study. The 1st set of 104 samples was selected for this study including samples positive at all levels for both IA-2Ab and ZnT8Ab, positive only for IA-2Ab, positive only for ZnT8Ab, and negative for both autoantibodies. The 2nd set of 180 samples was randomly selected samples from new-onset patients. The blood samples from these patients were collected within two weeks of their diabetes diagnoses. Patient ages ranged from 0.9 to 20 years (mean 11.0 years, median 11.6 years) for the 1st set and ranged from 0.9 to 17.8 years (mean 9.5, median 9.9) for the 2nd set. In addition, 10 prediabetics (1 to 10 years before diabetes onset) with either IA-2Ab and/or ZnT8Ab positive and 110 age-matched normal control samples from the general population were included in this study. Signed written consent forms were obtained from these participants, and the study was approved by the Institutional Review Board of the University of Colorado.

2.2. cDNA construct

Prior published studies (Wenzlau et al., 2007) indicate that the main epitopes of autoantibodies reacting with ZnT8 are located within the intracellular domain of the protein's C-terminus (amino acids 268 to 369). Three polymorphic variants of ZnT8 have been defined (Wenzlau et al., 2008b) with Arg325, Trp325, and Gln325. Arg325 and Trp325 were found to be the two major polymorphic forms of the ZnT8 autoantigen with a subset of individuals producing autoantibodies reacting with only one variant of ZnT8. A construct with the C-terminus of ZnT8 with Trp325 (W) and Arg325 (R) was linked with a hinge peptide of immunoglobulin by PCR followed by TA cloning to form the ZnT8 heterodimer, ZnT8WR. The IA2ic (IA-2 intracellular domain, amino acids 605 to 979) cDNA clone was kindly provided by Dr. Ezio Bonifacio of the Dresden University of Technology, Dresden, Germany. IA-2ic and ZnT8 heterodimer ZnT8WR were linked together with a linker peptide of 5 glycines to form a chimeric protein IA2–ZnT8WR with both autoantigens and the two common forms of ZnT8 (Fig. 1).

2.3. Radioassay

The fluid phase autoantibody radioassays utilized in the current study are identical to our standard islet autoantibody assays described elsewhere (Yu et al., 1996). In brief, radioactively-labeled autoantigen (IA-2, or ZnT8-R, or ZnT8-W, or IA2–ZnT8WR) was produced with an *in vitro* transcription/translation kit (Promega) and labeled with ^{35}S methionine (Amersham). Patient serum (2 μl) was incubated with 50 μl of assay buffer containing 20,000 cpm of the radio-labeled antigen overnight at 4 °C in a 96-well PCR plate, precipitated with 25 μl of 50% (v/v) Protein A-Sepharose (Amersham) in a 96-well filtration plate (Corning), washed on a vacuum folder (Millipore) for 2 cycles with 4 washings for each cycle, and directly counted on a Topcount (Perkin Elmer). The results are expressed as indices related to positive and negative control sera (index = $[CPM_{sample} - CPM_{negtive\ control}]/[CPM_{positive\ control} - CPM_{negative\ control}]$). The upper limits of normal controls were 0.015 for IA-2Ab (99th percentile of 100 normal controls); 0.040 for autoantibodies to ZnT8-R (ZnT8R-Ab) or to ZnT8-W (ZnT8W-Ab) (99th percentile of 100 normal controls); and 0.022 for autoantibodies to IA2–ZnT8WR (IA2–ZnT8Ab) (100th percentile of 110 normal controls). The coefficients of variation for intra- and inter-assays are 4.2% and 6.9%, respectively for IA-2Ab; 3.2% and 8.4%, respectively for ZnT8R-Ab or ZnT8W-Ab; and 4.5% and 9.6%, respectively for IA2–ZnT8Ab. In the most recent DASP workshop (2009), the sensitivity and specificity for our laboratory were 64% and 99%, respectively for IA-2Ab, and 54% and 99%, respectively for ZnT8Ab.

3. Results

The radioassay for autoantibodies to the chimeric protein antigen, IA2–ZnT8WR, and the radioassays for autoantibodies to their individual protein antigens were performed on serum samples from 110 normal control subjects, 284 patients with newly diagnosed diabetes, and 10 prediabetics, and the results from the radioassay for autoantibodies to the chimeric protein antigen, IA2–ZnT8WR, are shown in Fig. 2. Of the 294 samples, 231 were originally positive for either IA-2Ab and/or ZnT8Ab (111 both IA-2Ab and ZnT8Ab positive, 62 IA-2Ab positive only, 58 ZnT8Ab positive only and 63 both negative), and 100% of the 231 positive samples tested positive for autoantibodies to the chimeric antigen IA2–ZnT8WR with antibody levels above the 100th percentile of 110 normal control samples (>0.022). Of 180 randomly selected samples from new-onset diabetic patients, 24 out of 47 (51%) single autoantibody positive for current 3 standard screening autoantibodies (GAD65Ab, IA-2Ab, and mIAA) were ZnT8Ab positive and 6/19 all 3 screening autoantibody negative were ZnT8Ab positive. With IA2–ZnT8Ab assay replacing current IA-2Ab assay without

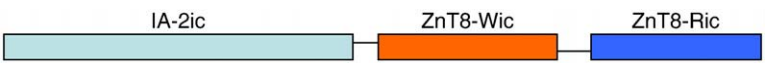

Fig. 1. The chimeric autoantigen, IA2–ZnT8WR, includes IA2ic (IA-2 intracellular domain from amino acids 605 to 979), ZnT8-Wic (ZnT8 intracellular domain from amino acids 268 to 369 with amino acid Trp at position 325), and ZnT8-Ric (ZnT8 intracellular domain from amino acids 268 to 369 with amino acid Arg at position 325). ZnT8-Wic is linked to ZnT8-Ric by a hinge peptide of immunoglobulin, and IA2ic is linked with ZnT8-Wic by 5 glycine amino acids.