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HIGH-THROUGHPUT RADIOASSAYS FOR AUTOANTIBODIES TO RECOMBINANT AUTOANTIGENS

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1. ABSTRACT

Advances in immunology, biochemistry, and molecular biology have combined to allow the development of a large series of autoantibody assays utilizing recombinantly produced autoantigens. Labeled target proteins can be readily produced by in vitro transcription and translation of relevant cloned cDNA. The assays are carried out in the fluid phase and for most assays are more specific and sensitive than ELISA based assays. For some antigens (e.g. insulin) though ELISA assays detect antibodies following immunization, workshops indicate they are almost worthless for the diagnosis and prediction of type 1A diabetes. This new generation of radioassays is usually carried out in 96-well microtiter filtration plates that allow high throughput. Given such assays, individuals at high risk for type 1A diabetes, celiac disease, and Addison's disease can now be readily identified.

2. INTRODUCTION

Autoimmune diseases are caused by a failure of the immune system to distinguish between host and foreign antigens. Self-tolerance can be defined as the lack of immune reactivity to autologous antigens. Autoimmunity is a circumvention of self-tolerance. Autoreactivity can occur through the humoral or the cellular effectors of the immune system. Autoantibodies may be the actual pathogenetic agents of the disease, the secondary consequence of tissue damage, or the "footprints" of an immunologic agent such as autoreactive T cells (1).

3. CATEGORIES OF AUTOANTIGENS

"Table 1" lists categories of autoantigens. Most autoimmune diseases are associated with immune responses directed against multiple autoantigens. Expression of an autoantigen can be restricted to a given

Table 1. Classification of antigen/ autoantigens of autoimmune diseases

Immunizing antigen	Molecule (exogenous or endogenous) that initiates immune response
Initiating autoantigen	Initial autoantigen to which immune response is directed
Perpetuating antigen/ autoantigen	A molecule whose presence maintains immune response(e.g. gliadin celiac disease)
"Suppressive" antigen/ autoantigen	A molecule which prevents/ blocks immune response

organ (typically in organ-specific autoimmune disease) or may be ubiquitous (often in non-organ-specific autoimmune disease but also for some autoantigens of organ-specific autoimmune disorders (e.g. transglutaminase)). It is unclear whether these autoimmune responses are driven by immunization to the self-proteins, to a cross-reactive non-self antigen, or are the result of a polyclonal cell activation of physiologically pre-existing autoreactive T and/ or B-lymphocytes. It appears that different diseases have different modes of activation. For example ingestion of the wheat protein gliadin is both necessary and sufficient to maintain the intestinal autoimmunity of celiac disease. With a diet that excludes wheat, autoantibodies to the self protein transglutaminase disappear and the intestine becomes normal. The actual initiation of disease is not as well understood as young children eat wheat for one or two years prior to the development of transglutaminase autoantibodies and celiac disease.

In most autoimmune disease, there exist circulating autoantibodies against various disease-associated autoantigens, and much effort has been devoted to the screening for these autoantigens. In the case of immune-mediated diabetes (type 1A), which is characterized by the chronic and progressive destruction of pancreatic islet beta cells (2), autoantibodies to islet cell cytoplasm (ICA) was initially identified with the immunohistochemical staining utilizing frozen sections of human pancreas more than 25 years ago (3, 4). This test has formed the basis for many studies of the natural history of the disease and the basis for several current large clinical trials of diabetes prevention. ICA can be detected in approximately 80 % of new-onset patients with type 1 diabetes and became the standard serological marker for specifying an autoimmune basis for type 1 diabetes (5, 6). Although ICA testing is a highly predictive marker for disease, the usefulness of ICA for routine screening of susceptible individuals or the general population is limited by the cumbersome nature of the ICA assay, difficulties of standardization, and problems with the consistency of the test. To develop sensitive, reproducible, and quantitative assays for autoantibodies to islet cell antigens, intensive efforts over subsequent decades have revealed the molecular identity of several of the antigen(s) of ICA reactivity by screening the islet cDNA libraries with ICA positive sera. Similar techniques were utilized to identify target antigens for autoimmune hepatitis. In some autoimmune diseases including Addison's disease, celiac disease, and pernicious anemia, target antigens against autoantibodies were also identified by peptide sequencing analysis of the immunoprecipitated protein, immunoblotting, or screening of cDNA expression libraries.

4. SPECIFIC DISEASES

4.1.Type 1A diabetes mellitus

Over the past decade, investigators have defined a large family of islet autoantigens. The determination of anti-islet autoantibodies has been revolutionized by the cloning of a series of islet autoantigens and the ease of current autoantibody radioassays. The isolation of autoantigens has made it possible to develop reliable and reproducible assays. For such radioassays many investigators simply take cloned cDNA of a given target autoantigen and *in vitro* transcribed and translated the cDNA to produced labeled autoantigen (7).

Glutamic acid decarboxylase 65 (GAD65) is a cytoplasmic enzyme expressed in all islet cells of man and a series of neuroendocrine tissues. It was the association of autoantibodies to GAD65 with stiffman syndrome, a rare neuromuscular disease characterized by muscle spasms, which first led to the identification of GAD65 as the elusive islet 64kD autoantigen (8).

The autoantigen ICA512 was discovered by Rabin and coworkers following the screening of an islet cDNA expression library with sera from patients with type 1 diabetes (9). The same molecule has been termed IA-2 by Lan and coworkers (10). Prior to the characterization of ICA512, Christie and coworkers had identified autoantibodies reacting with a 40kD and 37kD tryptic fragment of metabolically radiolabeled islets (11). It is now understood that the 40kD protein is ICA512/IA-2 and the 37kD molecule is a tryptic fragment of a molecule termed phogrin by Hutton and coworkers (12, 13), IA-2beta by Notkins and coworkers (14), and IAR by other investigators (15). ICA512/IA-2 and phogrin are both associated with neuroendocrine secretory granules (e.g. the islet granules containing insulin).

Four autoantigens as recombinant molecules (GAD65, ICA512/ IA-2, phogrin and insulin) are now readily available for biochemical autoantibody assays. In our laboratory autoantibodies to GAD65, ICA512/IA-2 and insulin are routinely measured in relatives of patients with type 1 diabetes. For detecting autoantibodies to GAD65, ICA512/IA-2 and phogrin, we are using a 96-well plate filtration technology and Top Counter microplate direct counting (16). In brief, *in vitro* translated ^{35}S-labeled protein is incubated with patients' serum (7 microliters for triplicate) at a 1: 25 dilution overnight at 4 ^{o}C, and the resulting immunocomplexes are precipitated with protein A-sepharose in the 96-well plate. After a washing step utilizing a vacuum-operated 96-well plate washer, radioactivity is determined directly in the 96-well plate with 96-well plate beta counter. The adaptation of the assay to a 96-well plate and semi-automated 96-well

Table 2. Recombinant anti-islet autoantibody assays

Antigen	Sensitivity (Specificity)	Comment
Insulin	40-95% (99%)	Inversely age of diabetes onset related
GAD65	70% (99%)	Predominantly age independent
ICA512/IA-2	60% (99%)	Islet protein tyrosine phosphatase
Phogrin/IA-2beta	55% (99%)	Autoantibodies predominantly subset of ICA512/IA-2 autoantibodies
Carboxypeptidase H	10% (99%)	Low sensitivity

counting allows a single person to analyze more than 40,000 samples per year. A similar assay format is used to measure insulin autoantibodies but with ^{125}I-insulin as the target autoantigen (17).

"Table 2" lists five islet autoantigens for which there currently exist autoantibody assays based upon recombinant DNA production of the autoantigen. Antibodies to carboxypeptidase H are too infrequent to contribute to a standard panel of autoantibodies (18). In addition to the antigens listed in "Table 2", there are a series of other potential additional autoantigens with partially characterized molecules, or characterized molecules where the assay format does not allow determination of antibodies for thousands of sera samples. It is likely that further characterization of these molecules, as well as potentially unknown molecules, or refinements in assay methodology will provide additional autoantigens of value in the diagnosis and prediction of type 1A diabetes.

Several different assays have been used to measure IAA, including formats of radiobinding and enzyme-linked immunosorbent assays (ELISA). As with ICA, several international workshops have addressed the standardization of IAA. The former workshops have shown that certain sera give markedly different results depending on assay methodology and have suggested that these discrepancies may be due to variation in the assay methodologies used: either the fluid-phase radiobinding assay or the solid-phase ELISA. In the fourth IAA workshop (19), the disease association of these autoantibodies as determined by radiobinding assay vs. that by ELISA was ascertained and it was concluded that fluid-phase radiobinding assay were more sensitive and specific than the solid-phase ELISA for measurement of these autoantibodies. Though ELISA formats could detect insulin antibodies of patients receiving insulin injections. Such assays failed to detect insulin autoantibodies associated with disease risk. The failure of ELISA formats in this case probably due to the antibodies of prediabetes being of high apparent affinity (10^{10}) and extremely low capacity (10^{-12}) (20).

4.2.Addison's disease

Idiopathic Addison's disease is the consequence of autoimmune destruction of adrenocortical steroid-producing cells (21). Adrenal cortex autoantibodies, antibodies that react with adrenal cortex, are detected in half to two thirds of patients with Addison's disease by indirect immunofluorescence using frozen adrenal sections (22-24). Although the role of these autoantibodies in the pathogenesis of Addison's disease is still unclear, the detection of a humoral immune response to adrenal autoantigens is an important disease marker useful in predicting Addison's disease. Betterle and coworkers found that 9 out of 10 adrenal cortex autoantibody-positive children developed overt Addison's disease and the remaining child had subclinical adrenal insufficiency after 3 to 121 months of follow-up (25). Winqvist and coworkers demonstrated that 21-hydroxylase, which is prominent in the zona glomerulosa of the adrenal cortex, is a major autoantigen in idiopathic Addison's disease (26). The 21-hydroxylase molecule belongs to the family of P450 cytochromes. It is an intracellular enzyme localized in the endoplasmic reticulum, depending on NADPH and a flavoprotein, P450 reductase, for its enzymatic activity. It converts 17-hydroxyprogesterone into 11-deoxycortisol in the glucocorticoid-synthesizing pathway and converts progesterone to 11-deoxycortisol in the mineraocorticoid pathway. Recent data document the strong association of autoantibodies to 21-hydroxylase with Addison's disease and the significantly increased prevalence of 21-hydroxylase autoantibodies among patients with type 1 diabetes (27-29). Autoantibodies to 21-hydroxylase were found in 86% of patients with Addison's disease and 100% of patients with Addison's disease of less than 20 years' duration (30). Approximately 1.5% of patients with type 1 diabetes without a known diagnosis of Addison's disease have these autoantibodies (31); this is increased to approximately 5% among the type 1 diabetic patients with the HLA-DQ2/DQ8 and DQA1*0501/DQB1*0301 genotype. The majority of 21-hydroxylase autoantibodies bind to the central and C-terminal parts of the molecule, which form a major conformational epitope (32). 21-hydroxylase autoantibodies correlate with progression to adrenal insufficiency in a similar way to adrenal cortex autoantibodies. In adults but not in children an association between HLA-DR3 and high titers of adrenal cortex autoantibodies and 21-hydroxylase autoantibodies has been reported.

Seroconversion of adrenal cortex autoantibodies or 21-hydroxylase autoantibodies usually precedes the clinical onset of Addison's disease (33). It is recommended that patients with organ-specific autoimmunity should be screened for the presence of these autoantibodies. A yearly hormonal evaluation of adrenal function in adrenal cortex autoantibody or 21-hydroxylase autoantibody-positive patients should allow early diagnosis and treatment.

4.3.Celiac disease

Celiac disease, a common cause of chronic malabsorption in children and is characterized by mucosal damage of the small intestine induced by gluten-containing foods, including wheat, rye, barly flours (34-36). Celiac

disease is a multisystem disorder with mucocutaneous, neurological and dental abnormalities in addition to intestinal lesions. The toxicity occurs in genetically-predisposed individuals (HMC class II, HLA-DR3-DQA1*0501-DQB1*0201 (DQ2)) and is produced by the gliadin-protein fraction of gluten. Homozygosity for DQA1*0501-DQB1*0201 alleles may predispose to an earlier disease onset and to more severe disease manifestations. This particular DQ Alpha/Beta heterodimer can be encoded in either cis or trans (DR3 haplotypes in cis; in trans with DR7 (DQB1*0201) plus DR5 (DQA1*0501)). DR3 is significantly increased in patients with celiac disease in all ethnic groups studied. The DR7 association with celiac disease has been more often reported from southern Europe, due to an increased frequency of the DR5/7 genotype in these populations. That gliadin induces immune system activation is supported by the presence of giadin-specific, DQ2-restricted T-cells in the mucosa of celiac disease patients and the detection of anti-gliadin, anti-reticulin, and anti-endomysial autoantibodies whose expression depends upon gliadin ingestion (37, 38). Removal of gliadin from the diet results not only in resolution of the intestinal lesions but also the disappearance of the autoantibodies. IgA antibodies to endomysium (EMA) (39), a structure of the smooth muscle connective tissue, are particularly specific indicators of celiac disease, suggesting that this structure contains one or more target autoantigens that play a role in the pathogenesis of the disease. Recently, Dieterich and coworkers identified tissue transglutaminase (tTG, glutamine Gamma-glutamyltransferase) as the endomysial autoantigen involved by the peptide sequence analysis of the protein specifically immunoprecipitated by IgA EMA positive sera (39). Transglutaminase is an enzyme of approximately 85kDa that catalyzes the cross-linking of proteins with glutamine. Gliadin predominantly contains glutamine amino acid (> 40%) and therefore is an excellent substrate for tissue transglutaminase. Gliadin can be coupled to tTG by the enzyme. It is hypothesized that the coupling of gliadin to tTG and deamidation of gliadin by tTG creates a novel T-cell epitope leading to autorecognition and disease.

The identification of tTG as an autoantigen made it possible to develop sensitive IgA and IgG radioassays for transglutaminase autoantibodies (TGAA) and evaluate it in patients with type 1 diabetes (40). From the screening of 598 patients with type 1 diabetes, 28 (4.7%) were originally positive for IgA EMA using a standard immunofluorescence assay. Among the 28 EMA-positive patients, the radioassay detected IgA TGAA in all subjects (100%) and IgG TGAA in 80%. Of these 28 patients, 14 have consented to intestinal biopsy and 10 biopsies were positive for celiac disease. All patients with TGAA level greater than index 0.75 had positive biopsy results. As reported previously, celiac disease in patients with type 1 diabetes is frequently asymptomatic and is only detected following autoantibody screening. The IgG TGAA radioassay may be useful among patients with IgA deficiency who have at least a ten-fold greater risk for celiac disease compared with general population. Transglutaminase autoantibodies associated with celiac disease are often of high titer. In limited comparisons, ELISA assays and radioassays have similar sensitivity and specificity (unpublished observation).

4.4.Pernicious anemia

Autoimmune gastritis (chronic gastritis type A) is characterized by a disturbed parietal cell function resulting in a reduced gastric acid production (41). In advanced stages of the disease, achlorhydria and mucosal atrophy in the gastric body and fundus are found. Apart from producing acid, the parietal cells also secrete intrinsic factor (42), the 60 kDa glycoprotein (43), which is essential for the vitamin B12 absorption. Deficiency of this vitamin results in pernicious anemia and neurological symptoms. The pathogenesis of autoimmune gastritis seems to involve an immune-mediated destruction of the parietal cells. The autoantibodies to a protein localized in the canalicula areas of the cell are found in 75 to 95 % of patients by an indirect immunofluorescence technique (44). Karlsson and coworkers identified that the major parietal cell antigen is the acid-producing enzyme, H^+, K^+-ATPase of the parietal cell (45). With an enzyme-linked immunosorbent assay (ELISA) using a membrane fraction of gastric mucose as antigens, autoantibodies to H^+, K^+-ATPase were detected in 93% of patients with pernicious anemia. There are two subunits of H^+, K^+-ATPase, alpha- and beta- subunit, and both subunits of H^+, K^+-ATPase are known to bind autoantibodies in sera from patients with autoimmune gastritis with pernicious anemia.

Using tunicamycin to inhibit glycosylation of the H^+, K^+-ATPase resulted in elimination of autoantibody binding in 98% of H^+, K^+-ATPase autoantibody-positive sera, indicating that the carbohydrates of the H^+, K^+-ATPase are necessary for autoantibody binding (46). Therefore, *in vitro* transcribed and translated recombinant protein which is not glycosylated would not be suitable for assay development.

4.5.Autoimmune hepatitis

Autoimmune hepatitis is an unresolving inflammation of the liver that is characterized by hyper Gamma-globulinemia, autoantibodies in serum, and the presence of at least periportal hepatitis (peacemeal necrosis) on histological examination (47). Autoimmune hapatitis is subclassified according to immunoserological markers into three types; type 1, type 2, and type 3 (48). Among these subtypes type 2 autoimmune hepatitis which afflicts mainly children (ages 2 - 14 years) is characterized by the presence of anti-LKM 1 (liver/kidney microsome type 1) autoantibodies and more commonly is associated with concurrent extrahepatic immunological diseases such as vitiligo, autoimmune thyroididtis, and type 1 diabetes mellitus (49). Recently Manns and coworkers identified the cytochrome monooxygenase P-450IID6 (CYP2D6) as a major target antigen of anti-LKM 1 autoantibodies (50). P-450IID6 (CYP2D6) is a 50 kDa microsomal enzyme that metabolizes at least 25 different drugs, including antihypertensive agents, beta blockers, antiarrythmic drugs, and antidepressants. Yamamoto and coworkers have developed the radioligand binding assay for CYP2D6

autoantibodies using *in vitro* translated [^{35}S]-CYP2D6 (51).

Table 3. Autoantibody radioassays based on the *in vitro* transcription/ translation of autoantigens

Worked	Did not work
GAD65	Proinsulin
ICA512/ IA-2	ICA69
Phogrin/ IA-2beta	H^+, K^+-ATPase
Carboxypeptidase H	
21-hydroxylase	
CYP2D6	
Transglutaminase	

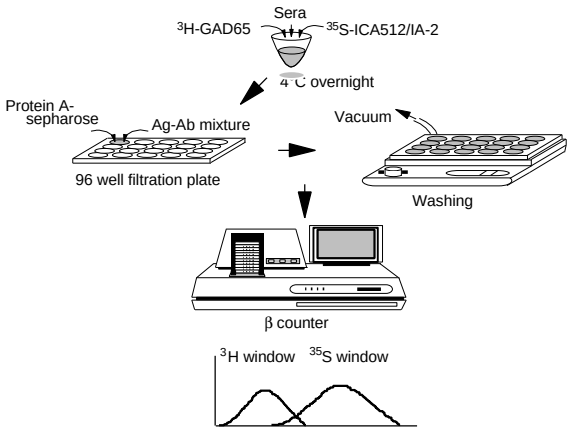

Figure 1. Schematic method for combined GAD65 and ICA512/IA-2 autoantibody radioassay.

They detected the CYP2D6 autoantibodies in 100 % (40/40) of anti-LKM 1 autoantibody-positive patients with autoimmune hepatitis type 2, and 7 % (1/14) of sera from patients with primary biliary cirrhosis. We evaluated the prevalence of CYP2D6 autoantibodies in more than 250 patients with type 1 diabetes and found that none of these sera were positive for this autoantibodies, indicating the low prevalence of autoimmune hepatitis type 2 in type 1 diabetes.

4.6.Radioassay Summary

"Table 3" lists *in vitro* transcribed and translated recombinant autoantigens which we have successfully utilized for disease diagnosis and prediction. In addition three molecules where *in vitro* transcription/ translation based assays have not been useful are for proinsulin, a molecule termed ICA69 (52), and H^+, K^+-ATPase (46). For H^+, K^+-ATPase glycosylation of the antigen may be essential. For proinsulin it is likely that the conditions for proper folding are not present in our *in vitro* system. This is also likely with ICA69.

5. PRODUCING "BETTER" ASSAYS FOR KNOWN AUTOANTIGENS

We have recently developed a combined ICA512/ IA-2, GAD65 autoantibody radioassay utilizing [^{3}H]-labeled GAD65 and [^{35}S]-labeled ICA512/ IA-2 which allows simultaneous detection and discrimination of both autoantibody specificities (16). In vitro translated [^{3}H]-GAD65 and [^{35}S]-ICA512/ IA-2 were mixed and incubated with serum in a tube and the radioactivity was counted in a

96 well-plate with channel windows set for each radionucleotide after protein-A sepharose precipitation ("Figure 1"). The combined assay gave essentially identical results to those obtained in the single radioassays, and detected either antibody in more than 90% of patients with new-onset diabetes and prediabetic relatives. The simplicity of this assay with dual determination of the two antibodies utilizing 7 microliters of sera, 96-well membrane separation of autoantibody bound labeled autoantigen, and 96-well beta counting facilitates the rapid screening of thousands of samples. Investigators have readily shared antigen clones with other clinical investigators and thus the GAD65 and ICA512/IA-2 autoantibody assays have been readily adopted in laboratories on six continents.

Though insulin was the first autoantigen biochemically characterized, the assay for anti-insulin autoantibodies (IAA) was much less convenient compared to assays for GAD65 and ICA512/IA-2. The original IAA assay utilizes 600 microliters of sera for duplicate determinations with and without competition with unlabeled insulin (53, 54). The conventional IAA assay is performed in centrifuge tubes and is thus labor intensive. It has been important to improve the IAA assay as IAA are one of the first autoantibodies to appear in a prediabetic individual and provides the most sensitive assay for detecting children less than age 10 developing type 1 diabetes (55). Attempts to measure IAA by standard ELISA techniques led to assays which could detect anti-insulin antibodies following subcutaneous insulin therapy but could not detect the autoantibodies of prediabetic and new onset patients with type 1 diabetes (19). Recently, Williams and coworkers have developed a novel small volume assay (less than 50 microliters of serum) for IAA which is suitable for screening large number of samples (56). In new-onset patients with type 1 diabetes and the first degree relatives of patients with type 1 diabetes, the results were highly concordant and IAA levels correlated well with the conventional IAA assay. We have modified the Williams assay so that it can be performed in 96 well filtration plates with beta counting similar to the GAD65 and ICA512/IA-2 autoantibody assays (17).

6. AUTOANTIBODY EPITOPES AND IGG SUBCLASS

GAD autoantibodies are important serological marker of the autoimmune process of type1 diabetes because they are present in the majority of individuals with new-onset diabetes and in individuals with the prediabetic stage of the disease. However, GAD autoantibodies are also found in some individuals without type 1 diabetes, including patients with stiffman syndrome or autoimmune polyendocrine syndrome type 1. These individuals have a low risk of developing type 1 diabetes. Powers and coworkers have demonstrated that, in type 1 diabetic sera, GAD65 autoantibodies are predominantly directed to conformation-dependent epitopes located to the middle (aa 240-359) and carboxy-terminal (aa 453-569) regions of the GAD65. In contrast, sera from patients with stiffman syndrome recognize the epitopes located to the amino-, middle-, and carboxy third of the GAD65 protein and bind with denatured GAD (57-59).

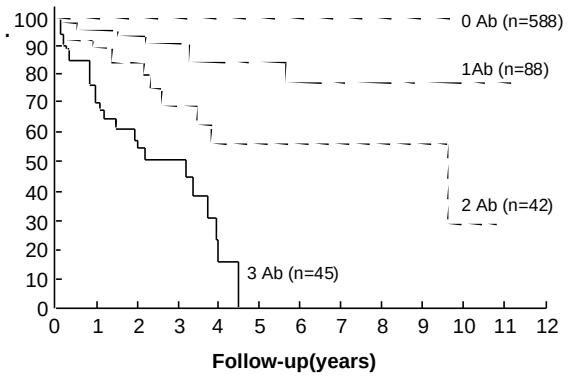

Figure 2. Progression to diabetes of first degree relatives subdivided by the number of "biochemical" anti-islet autoantibodies at first determination. The Y axis indicates the percentage of non-diabetic first-degree relatives of type 1 diabetes.

Autoantibodies to ICA512/IA-2 are present in 60-70% of prediabetic relatives and new-onset patients with type 1 diabetes. With a series of ICA512/IA-2 fragments and chimeric ICA512/phogrin molecules others and we analyzed the dominant epitopes recognized by autoantibodies to ICA512/IA-2 (16, 60, 61). The major epitopes recognized by sera from type 1 diabetic patients are located within aa 687-979 of ICA512/IA-2, aa 605-682 of the juxtamembrane region, and a conformational epitope associated with carboxy-terminal 31 amino acids (aa 949-979) of ICA512/IA-2 which was recognized by one-third of sera. In first-degree relatives of type 1 diabetes who have progressed to type 1 diabetes, we have reported the inter- and intramolecular epitope spreading of autoantibodies to islet autoantigens (62, 63). The appearances of humoral autoimmunity initially appear limited to insulin and GAD65 followed by a later response to ICA512/IA-2 (intermolecular epitope spreading). Furthermore, in 13 relatives who have progressed to type 1 diabetes 5 exhibited intramolecular epitope spreading of ICA512/IA-2 autoantibodies during the preclinical phase. These studies are consistent with the hypothesis that autoimmunity directed toward these molecules may be the result of chronic autoimmune destruction of the Beta-cell and attendant presentation and immune recognition of autoantigens by elements of the inflammatory infiltrate. Such a mechanism would be consistent with the seeming paradox that disease-specific autoantibodies are associated with an antigen that has a broader tissue distribution than the pancreatic islet.

The nature of an immune response is thought to reflect a balance of antigen-stimulated T-helper $CD4^+$ cell subsets. T-helper 1 (Th1) cells secrete IFN-gamma, IL-2 and TNF-beta and mediate cellular immunity or delayed-type hypersensitivity; T-helper 2 (Th2) cells secrete IL-4, -5, -6 and –10 required for B cell, mast cell and eosinophil differentiation, thereby mediate humoral immunity and allergy (64-66). Studies in animal models of type 1 diabetes have shown that destructive autoimmunity is mediated through a Th1-dominat autoimmune response.

This has been suggested by some to arise relatively late in the disease process after a latent period of non-aggressive Th2 islet autoimmunity. In the mouse Th1 response are associated with the generation of IgG2a and IgG3 subclass antibodies and Th2 response with IgG1 (67, 68). Based on the functional properties it is suggested that IgG1/IgG3 and IgG2/IgG4 in humans might be the equivalent, respectively, of IgG2a/IgG3 and IgG1 in mice. In patients with type 1 diabetes studies of IgG subclass of ICA have revealed heterogeneity, with a predominance of IgG1. Couper and coworkers analyzed the IgG subclass response to GAD in new-onset patients with type 1 diabetes and in at-risk first-degree relatives of patients with type 1 diabetes (69). They reported that, in the new-onset patients, IgG1 and/or IgG3 antibodies to GAD were significantly more frequent than IgG4 or IgG4/IgG2, and high frequency of IgG2 and/or IgG4 antibodies were observed in ICA-positive relatives who had not progressed to diabetes. Bonifacio and coworkers measured IgG subclass antibodies to insulin, GAD, and ICA512/IA-2 sequentially from birth to diabetes onset in offsprings of patients with type 1 diabetes (70). They reported that autoantibody appearance was characterized by IgG1 peak response to one or more autoantigens and that the presence of IgG1-restricted response to ICA512/IA-2 was associated with diabetes development. These studies suggest that type 1 diabetes has an early acute destructive phase of Beta cell autoimmunity which may be regulated and that the IgG subclass antibodies to islet antigens may define risk for progression to type 1 diabetes.

7. COMBINATORIAL PREDICTION OF TYPE 1 DIABETES

The long prodromal phase preceding the onset of clinical symptoms in type 1 diabetes suggests that it should be possible to predict and design trials for the prevention of the disease (71, 72). To date, the best autoantibody predictor of high diabetes risk is the expression of multiple "biochemically" determined autoantibodies (73). Figure 2 illustrates the progression to diabetes amongst a series of more than 600 relatives of patients with type 1 diabetes subdivided by the number of autoantibodies expressed testing for GAD65, insulin and ICA512/ IA-2 autoantibodies. Only one relative of more than 500 expressing none of these three autoantibodies progressed to diabetes. Expression of a single autoantibody was associated with an approximate 20% risk of diabetes with ten years of follow up and it is likely that many relatives expressing a single autoantibody will never progress to diabetes (73). Expression of multiple autoantibodies was associated with a very high risk of progression. The "combinatorial" analysis allowing $\geq$2 autoantibodies to be defined, independent of which two autoantibodies are expressed and gives approximately an 80% sensitivity for progression to diabetes with very high specificity. Less than 1/300 individuals from the general population express $\geq$2 autoantibodies, indicating expression of multiple autoantibodies approaches the risk of type 1 diabetes.

It has been known that positive predictive value for developing disease depends on the disease incidence

Table 4. Estimates of sensitivity, specificity, and positive predictive value in first-degree relatives of type 1 diabetes and general population

		First-degree relatives		General population	
	Sensitivity	Specificity	Positive predictive value	Specificity	Positive predictive value
$\geq$ 1Ab	97.8%	87%	28%	97%	9.6%
$\geq$ 2Ab	78.0%	97%	56%	99.7%	46%

*Sensitivity was calculated from the data in prediabetics and new-onset patients with type 1 diabetes $\geq$ 1Ab, $\geq$ 2Ab; $\geq$ 1, $\geq$ 2 autoantibody positive amongst autoantibodies to insulin, GAD65, and ICA512/IA-2

(74, 75). The usefulness of screening of the general population and of unaffected relatives of patients with type 1 diabetes for autoantibodies depends on the accuracy of the available screening tests. Both the prevalence with which a disease develops in a given population and the specificity and sensitivity of the assays affect the clinical applicability of screening programs. In the United States, the incidence of type 1 diabetes among children age less than 19 years is 18.2 per 100,000/ year (76). Applying this rate to the U.S. population, an estimated 13,000 new cases arise each year in persons less than age 19 years. Most studies of prediabetic subjects have involved the screening of first-degree relatives of probands with type 1 diabetes, rather than the general population. However, only about 10% of new cases of type 1 diabetes have an affected relative. This indicates that the general population eventually will need to be screened if an effective intervention is to have a major impact on the incidence of the disease. As described above, expression of multiple autoantibody specificities is the best predictor of diabetes risk and combinatorial analysis of autoantibody expression is both more sensitive and specific than ICA testing. The sensitivity of autoantibody expression amongst relatives developing diabetes is approximately 98% for any single autoantibody ($\geq$ 1Ab of autoantibodies to insulin, GAD65 and ICA512/IA-2) and 78% for more than two autoantibodies. Bayes' theorem assumes equal diagnostic specificity and variable prior disease probability while it appears that relatives of patients with type 1 diabetes more often express autoantibodies despite low risk for progression to diabetes (approximately 13% for $\geq$1Ab for ICA-negative relatives and 1-3% for $\geq$2Ab for ICA-negative relatives) than individuals in the general population (approximately 3% for $\geq$1Ab for ICA-negative subjects and 0.3% for $\geq$2Ab for ICA-negative subjects). When combinatorial autoantibody tests are applied to first-degree relatives with type 1 diabetes, where the prevalence of clinical diabetes is 1/20, then the positive predictive values are 28% for $\geq$1Ab and 56% for $\geq$2Ab, respectively. If those same criteria were applied to the general population where the disease prevalence is 1/300, then the positive predictive values would be 9.6% for $\geq$1Ab and 46% for $\geq$2Ab, respectively. It is likely that presence of multiple autoantibodies will be as predictive in the general population as amongst first degree relatives and that the combinatorial autoantibody assay will have to be used to accurately identify individuals at risk for type 1 diabetes ("Table 4").

8. CONCLUSION

It is likely that many tests for autoantibodies can be improved (increased sensitivity and specificity) by adopting fluid phase assays utilizing recombinant autoantigens. Documenting such improvements will likely require "blinded" workshops with sera from defined population and direct comparison of different assays. The field of type 1 diabetes has greatly benefitted from workshops where investigators analyzed in blind sera from relevant populations and assays were compared. With such comparisons specific and sensitive assay can be rapidly introduced into clinical practice.

9. ACKNOWLEDGEMENTS

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