Turkish Journal of Endocrinology and Metabolism, (1998) 4 : 225-229 **ORIGINAL ARTICLE**

Comparison of Indirect Immunofluorescence and Peroxidase-Labeled Protein a Methods in Insulin-Dependent Diabetes Mellitus

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Islet cell antibodies (ICA) have been proven to be important in the prediction and diagnosis of Type 1 diabetes mellitus. Indirect immunfluorescence (IF) technique has been used for ICA detection since 1974; but researchers have tried to establish a more practicable and convenient method. The peroxidase-labeled protein A (POPA) method has some advantages over conventional IF; for example it is cheaper and reevaluable for over 5 years. Also human pancreas obtained from donors of all blood groups and all ages can be used as a substrate in this method. In this study, we have tested 66 sera of insulin-dependent diabetes mellitus (IDDM) patients in ethe arly clinical stage (< 6 months), 30 sera of slowly progressive Type 1 diabetic (SPIDDM) patients with both IF and POPA techniques. In the control groups, there were 8 sera of first degree non-diabetic relatives of IDDM patients and 10 non-diabetic healthy people. Although, there were no statistical differences between these two methods (p>0.05) if the IF method is accepted as gold standard; sensitivity and specificity of the POPA method were 80% and 60% respectively. Its positive predictive value was 76% and negative predictive value was found to be 72%.

KEY WORDS Indirect Immunofluorescence Method, Peroxidase-Labeled Protein a Method, Type 1 Diabetes Mellitus

Introduction

Islet cell antibodies (ICA) were first detected in patients suffering from Schmidt's syndrome (polyendocrine autoimmunity) by Bottazzo et al (1). in the mid-1970's. Since patients with this syndrome were frequently noticed to develop insulin-dependent diabetes mellitus (IDDM), ICA were then rapidly demonstrated in sera of newly diagnosed diabetic children (2). ICA may also be found prior to the diagnosis of IDDM up to 9 years before IDDM is diagnosed. ICA are of the IgG type; while ICA IgM are detected rarely perhaps due to a long subclinical history of the disease. Proteins such as glutamic acid decarboxylase as well as monosialogangliosides in islet cell cytoplasm are suspected but antigens of ICA remain to be established (3). In the majority of IDDM patients, ICA disappear within 6 months to 3 years after clinical onset of the disease (4). Detection of ICA in unaffected relatives of IDDM patients in the period preceding the clinical diabetes led to the possibility that ICA may be used as a predicting marker of impending IDDM (5,6). Moreover the presence of ICA in patients with non-obese NIDDM (non-insulin dependent diabetes mellitus) implies a high risk of future insulin dependency (7). ICA were first revealed with indirect immunofluorescence (IF) test on unfixed cryostat sections of human pancreas (1). Since that time, many efforts have been made to modify the test procedure in order to increase the sensitivity of the standard method (8). An

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enzyme-linked immunosorbent assay (ELISA) was established for the detection of islet cell antibodies in human sera. The ICA-ELISA provides a highly standardised and reproducible test method for IDDM (9). We previously compared ICA-ELISA with ICA-IF method, and found that ICA-ELISA could give about 70% concordance to IF (Satman et al. 1991, unpublished results). Fluorescent conjugates of antibodies to the complement components C3, C1q, and C4 are now freely available so that the complement-fixation test (CF) can be done specifically on tissue sections, and endocrine-cell antibodies can be classified accurately according to their ability to fix complement. Appearance of CF-ICA in the circulation may be related to the clinical onset of diabetes (10). Since Mr 64,000 (64K) islet cell protein, later recognised as glutamate decarboxylase (GAD), is a major target antigen in IDDM; immunoprecipitating antibodies to this protein have been found in 80% of newly-diagnosed cases, and they have also been detected in the pre-diabetic period in first degree relatives of IDDM patients who later developed diabetes (11). Srikanta et al. have observed that use of an optimal dilution of fluorescent-conjugated protein A as a "non-antibody" chemical probe to detect human immunoglobulin binding to the pancreatic islet eliminates many of the problems encountered in the assay and facilitates standardization (12).

And finally, Takahashi et al. developed a new method of detecting ICA using peroxidase-labeled protein A (POPA), and determined the prevalence of ICA in IDDM patients in Japan in 1986 (13). In this study, we tried to compare the POPA method with the standard IF method in ICA determinations on patients with different stages of IDDM.

Materials and Methods

Subjects

Patients, attending the Diabetes Research and Application Unit, were selected according to the criteria of WHO (World Health Organisation) established in 1985 (14). Mean age of early clinical stage patients was 16.4 ± 9.9 and of SPIDDM patients was 43.2 ± 11.4. It was 18.1 ± 10.1 for first degree non-diabetic relatives of IDDM patients and 22 ± 10 for non-diabetic healthy group. The total numbeı of patients was 96 and of control persons was 18 (Table 1).

Table 1. General characteristics of groups examined in this study.

Patient Groups	Duration of diabetes	No	AGE (year) (mean±SD)
IDDM			
(Early clinical stage)	<6 monts	66	16.4±9.9
Slowly progressive IDDM	<5 year	30	43.2±11.4
Control 1: First degree nondiabetic relatives of IDDM patients	-	8	18.1±10.1
Control 2: Nondiabetic healthy group	-	10	22±10
Total		114	

Methods

ICA was detected by two methods, the IF methoc and immunochemical staining with POPA. In the IF method (2); unfixed, 5 micrometers (μm) cryostat sections of human pancreas obtained from cadaver donors with blood group 0 were stored at -70°C; sections were dried for a few minutes before use. Sections were incubated with sera for 30 minutes at room temperature, and washed with phosphate-buffer saline (PBS, pH: 7.2) for 20 minutes. FITC-conjugated antihuman sheep IgGAM (The Binding Site Ltd.) was applied for 20 minutes in 1/50 dilution. Then the sections were washed again Slides were mounted in glycerol/PBS buffer, pH: 8.6 and examined under a fluorescence microscope fitted with epiillumination.

In the POPA method (13), fresh frozen sections of the pancreas were washed with 0.01 mol/ L. PBS (pH: 7.2) containing 2% bovine serum albumin (BSA). The pancreatic sections were applied with 50 μl serum and they were incubated at room temperature for 60 min. After incubation the sections were washed in PBS three times. The pancreatic sections were then rinsed with POPA (20 μg/ ml, 50 μl) and were incubated again at room temperature for 30 min. After being washed with PBS, they were stained with Karnovsky's diaminobenzidine (DAB) solution for 3 min. The staining sections were washed, dehydrated with alcohol, plated and observed under light microscopy. In addition,

the same sections were first stained with haematoxylin and eosin (HE) to verify the presence of Langerhans islets.

Normal human pancreata were obtained from 3 male cadavers (ages; 46, 65, and 22 years) with blood group 0. The fresh tissues were frozen in isopentane cooled in liquid nitrogen and stored at -70°C.

ICA titres. The end-point titre was defined as the highest dilution of serum giving a positive stained reaction in both IF and POPA methods. End-point titres were then transformed into JDF u (Juvenile Diabetes Foundation units) using a formula derived from $\log_2$ of end-point titres of standard sera. ICA ≥ 10 JDF u was defined as positive.

Statistics. "Student's t test" on paired samples has been used for comparison of the two methods. The sensitivity, specificity, and positive and negative predictive values of the POPA method have been calculated against the IF method.

Results

As shown in Figure 1, cells of Langerhans islets were stained brown with serum containing anti-islet cell antibody by the POPA method. Figure 2 represents a positive reaction by IF method. In this study, 80.9% of early clinical stage patients were detected as positive and 36.6% of SP cases were positive by IF. In first degree relatives 62.5% were positive and in the healthy group there were no positive results. By POPA, results were 54%, 43%, and 25%, respectively and in the healthy group, no positive result was detected (Table 2).

In these experiments, when IF method was accepted as gold standard the sensitivity of POPA method was calculated as 80% and specificity as 60%. Positive predictive value of POPA method was 76% and negative predictive value was 72%. According to these results, there were no differences between these two methods statistically (P>0,05). Positive sera have been diluted with PBS from 1/2 to 1/ 256 in the two methods and end-point titres were determined very similar to each other being the same in 30% tests. Remaining sera were defined as either 1-2 dilution less (35%) or one dilution more (35%) on POPA.

Figure 1. Positive POPA staining reaction of ICA on human pancreas. The cytoplasm of the Langerhans islet cells is stained brown (x20).

Figure 2. Positive IF staining reaction of ICA on human pancreas. The cytoplasm of the Langerhans islet cells is stained green. Fluorescence intensity score is 4-5 (x20).

Table 2. Results obtained from ICA tests with IF and POPA methods.

Patient Groups	IF		POPA	
	Positive	Negative	Positive	Negative
EARLY IDDM	53 (80.9%)	13 (19%)	36 (54%)	30 (46%)
SPIDDM	11 (36.6%)	19 (63%)	13 (43%)	17 (56.6%)
AT RISK*	5 (62.5%)	3 (37.5%)	2 (25%)	6 (75%)
HEALTHY	0 (0%)	10 (100%)	0 (0%)	10 (100%)

Discussion

The presence of antibodies to islet cell antigens in sera from patients with IDDM was first described by Bottazzo et al (2) and, except for minor modifications, the same assay is still in use today (15). Two major limitations of ICA must be remembered when evaluating results. Even with standardised

procedures for preparing the pancreas, there is still considerable variability between pancreata in how well they work in the ICA assay (16) This can result in variability between laboratories that use different pancreas specimens. Individual laboratories also frequently experience variability when they are forced to change from one pancreas to another. The second major difficulty with current ICA methodology is that the reading of the immunofluorescence is subjective and therefore prone to variability between readers.

Since ICA were first described, numerous methodological modifications to the standard IF assays have been proposed. Because an assessment of inter-assay comparability was essential for the validity of ICA in diabetes research, accordingly, four International Workshops have been held. The first showed a large scatter of results between laboratories, and suggested that the availability of reference sera would allow laboratories to express ICA in arbitrary, but common units. A set of reference standard sera were distributed and tested in different laboratories, and based on standard curves derived from end-point titer of each serum, results were defined as Juvenile Diabetes Foundation (JDF) units and tested in the stage II Workshop. The use of standard curves constructed from this standard improved precision and concordance between laboratories. The stage III workshop was designed so as to (1) assess the precision, and (2) begin studies on the specificity (negativity in health) and sensitivity (positivity in disease) of the ICA assays (17).

The other disadvantages of IF method are difficulty in obtaining human pancreas from young and blood group 0 donors, high background and fading of slides. An alternative procedure for ICA determinations has been investigated for many years. Nakane and Kawaoi (18), conjugated horseradish peroxidase (HRPO) and protein A (PA) by periodate method in 1974. Ternynck and Avrameas (19) used p-benzoquinone in 1977 and Molin et al (20). performed this conjugation with 25% glutaraldehyde. In our initial experiments we conjugated HRPO and PA with 25% glutaraldehyde by using chemical coupling and purified it by colon chromatography obtaining POPA conjugate (21). Then we used POPA as a second antibody in ICA experiments but had to perform some modifications in order to reduce background and have a high quality preparation. For example we tried duration of acetone fixation, serum incubation, POPA and chromogen (DAB) incubations as different choices. Also addition of 30% hydrogen peroxide(H_2O_2) into DAB solution was tried with 1,2,4,5 µl and optimum concentration was provided with 2 µl. Marshall et al (22). used protein A-peroxidase/ diaminobenzidine secondary antibody system with human or rat pancreas for the measurement of ICA. Specific ICA binding values with human and rat pancreas were similar. Kawasaki et al (23). detected ICA by POPA method (13) on serial dilutions of serum samples and their laboratory participated in the ICA Proficiency Test under the auspices of the Immunology and Diabetes Workshop. Takahashi et al (13). performed the comparison of IF and POPA methods on 52 IDDM patients and 100 control subjects. Three sera from IDDM patients showed positive reaction only by the POPA method, and titres of these sera were 1x One serum from a control subject showed positive staining only by the IF method. ICA were detected in 100% of patients with less than 0.5 years duration, 50% of 0.5-1 years duration, 44% of 1-5 years duration and 13% of patients with >5 years duration by the POPA method.

In the present study, we have tested ICA of totally 114 sera, 66 of which were obtained from an early clinical stage and 30 were from SP patients. In the control groups, there were 8 sera from first degree relatives of IDDM patients and also 10 non-diabetic healthy people, and then we compared the results by IF and POPA methods. We have not determined any statistical difference between these two methods except for early IDDM patients.

POPA method has the following advantages: (1) any human pancreas can be used regardless of donor's age and blood type, (2) sections can be stored for more than 5 years, (3) light microscope is used instead of fluorescence microscope, (4) background is low, and finally (5) it is cheaper than IF. In conclusion, POPA method with 80% sensitivity and 60 % specificity may be substituted for IF method because of the above advantages

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