ORIGINAL ARTICLE

Autoimmune Markers in Young Malaysian Patients With Type 1 Diabetes Mellitus

W M Wan Nazaimoon[1], K Nor Azmi[2], R Rasat[2], I S Ismail[3], M Singaraveloo[4], W B Wan Mohamad[5], R Letchuman[6], I H Sheriff[2], I Faridah[2], B A K Khalid[2], [1]Division of Endocrinology, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, [2]Faculty of Medicine, Universiti Kebangsaan Malaysia, [3]Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, [4]Hospital Sultanah Aminah, Johor Bharu, Johore, [5]Faculty of Medicine, Universiti Sains Malaysia, Kubang Kerian, Kelantan, [6]Hospital Kota Bharu.

Summary

This study determined the prevalence and significance of autoantibodies to GAD65 (GAD Ab), insulin (IAA), tyrosine-like phosphatase (IA2) and islet-cell (ICA) in a group of 213 young Malaysian Type 1 diabetics, diagnosed before the age of 40 years. Venous blood was taken at fasting, and at 6 minutes post-glucagon (1mg i.v.). IAA was detected in 47.4%, GAD Ab in 33.8%, IA2 in 8.9% and ICA in 1.4% of the subjects. When based on post-glucagon C-peptide level of 600pmol/L, 172 (80.7%) patients had inadequate pancreatic reserve, while the remainder 41(19.3%) showed normal response. The autoantibodies, either alone or in combination, were detectable in both groups of patients; higher prevalence in those with poor or no ß-cell function (73.3% versus 46.3%, p=0.0001). Although the prevalence of GAD Ab was highest in newly diagnosed patients (<5 years), unlike IA2 and ICA, the marker remained detectable in 24 - 25% of those patients with long-standing disease. Nineteen patients could probably belong to the "latent autoimmune diabetes in adults (LADA)" subset, where pancreatic reserve was adequate but patients had detectable autoantibodies and insulin-requiring. On the other hand, 68 of the 213 patients (32%) were seronegative, but presented with near or total ß-cell destruction. Thus, as has also been suggested by others, there is indeed etiological differences between the Asian and the Caucasian Type 1 diabetics, and, there is also the possibility that other, but unknown autoantigens are involved in causing the pancreatic damage.

Key Words: Glutamic acid decarboxylase antibodies, Insulin autoantibodies, C-peptide

Introduction

Autoantibodies to islet cells (ICA), insulin (IAA) and glutamic acid decarboxylase (GAD65) are immune markers implicated to play important roles in the pathogenesis of Type 1 diabetes mellitus (Type 1 DM). Of these, ICA and IAA are widely used, with the prevalence rates of about 84% and 43% respectively amongst newly diagnosed Type 1 diabetics[1,2]. However, the measurements of these antibodies are difficult to standardise[3,4] and IAA have been reported to be present in Type 1 diabetics even before the beginning of insulin treatment[5]. In contrast, GAD65 antibodies (GAD Ab) can be easily quantitated[6], and have high diagnostic sensitivity and specificity at around 70 - 80% and 96-98% respectively[7,8]. Whilst ICA titres are often transient[9,10] GAD Ab has been shown to be persistent and precede clinical manifestation by up to 10 years[11]. GAD Ab has also been suggested to be highly predictive, identifying the 'Latent autoimmune diabetes in adults' (LADA) subset amongst the Type 2

diabetics[12,13]. On the other hand, the antibodies to tyrosine-like phosphatase (IA2) is yet another immune marker thought to be also involved in ß-cell destruction, although its value as predictor for Type 1 DM is enhanced only when considered in combination with the other autoantibodies[14,15].

However, a number of studies have showed that the pattern of appearance of these immune markers among Asian diabetic subjects were different from that of the Caucasians[16,17]. This study was therefore carried out to determine the prevalence of GAD Ab, IA2, IAA and ICA in young Malaysian Type 1 diabetic patients and to evaluate their clinical significance in the diagnosis as compared to the measurement of C-peptide response to glucagon stimulation.

Materials and Methods

A total of 213 Type 1 (91 males and 122 females, aged 2 months to 50 years) from several centres representing different geographical parts of Malaysia were recruited for this study. Patients were diagnosed to be Type 1 diabetics based on clinical features, insulin requirements, presence of ketones or ketoacidosis and biochemically, according to WHO recommendations. All patients were diagnosed before the age of 40 years and have had the disease for not more than 10 years. Following an overnight fast of 8 - 10 hours, each patient was bled for fasting sample, then given 1mg glucagon i.v. and 6 minutes later, rebled for post-glucagon C-peptide level. Sera were stored at -20°C until analysed. All patients gave informed written consent prior to the study. The study protocol was approved by the Ethics Committees of all participating hospitals.

GAD65 and IA2 antibodies were measured by RIA using kits purchased from RSR Ltd, United Kingdom. Briefly, test serum samples were first incubated with ^{125}I-labelled human recombinant of either GAD65 or IA2, followed by the addition of solid phase protein A to precipitate the bound complexes. The intra-assay coefficient of variations (CVs) of GAD65 at 4 and 25U/ml were 1.7 and 2.3% respectively, while the corresponding inter-assay CVs were 3.4 and 5.8% respectively. Based on the fact that the mean GAD Ab level determined in 202 young healthy blood donors were between 0 - 0.8U/ml, a sample was considered to be seropositive to GAD Ab when the level was equal to or above 2U/ml. The intra-assay CVs of IA2 at 4.8 and 25.6U/ml were 4.1 and 1.5% respectively, while the corresponding inter-assay CVs were 4.1 and 9.3% respectively.

The ELISA kits for ICA and IAA were purchased from DRG International, Inc. U.S.A. The assays involved incubating the test samples with human insulin or pancreatic antigens which had been immobilized onto microwells. Following several washing steps, the bound antibodies were detected using HRP-labelled goat antibody. Two quality control sera (positive and negative) provided with each test kit were used to monitor the validity of the results. In order to set the cut-off value for positivity, 50 serum samples obtained from normal subjects were analysed for the autoantibodies. Mean OD (±SD) reading of normal samples were 0.52±0.02 and 0.34±0.01 for ICA and IAA respectively. A patient was thus taken to be seropositive for ICA or IAA when the sample OD reading was above 3 SD that of the mean OD obtained for the normal subjects.

Statistical Analysis

All analyses were done using the SAS program. Differences in frequencies were tested using the Chi-squared or Fisher's exact test, wherever appropriate. Comparison between groups was made using Mann-Whitney U test.

Results

The overall prevalence of GAD65, IA2, IAA and ICA amongst the Type 1 diabetic subjects are shown in Table I. IAA was the most commonly detected antibodies, found in 47.4% of the patients, followed by GAD65 in 33.8% and IA2 in only 8.9% of the patients. In contrast, there were only 3 patients (1.4%) found to be positive for ICA. No significant gender preference in the distribution of these antibodies was observed. The characteristics of the patients based on their C-peptide response to glucagon stimulation and their autoimmune status are as shown in Table II. Of the 213 patients, 172 (80.7%) showed inadequate C-peptide response of less than 600 pmol/L, while 41 (19.3%) patients, though clinically diagnosed to be Type I, actually had normal

stimulated C-peptide levels. Autoimmune markers, GAD65, IAA, IA2 and ICA, either alone or in combination, were detectable in both groups of patients, although the prevalence was higher in those with poor or no ß-cell function (73.3% versus 46.3%, p=0.0001, Table II). Of those 19 patients with adequate pancreatic reserve but had detectable autoantibodies, all (100%) were positive for IAA, 4 (21%) for GAD Ab and only one (5%) for ICA. Patients who showed inadequate response were characterised by younger age of disease onset and significantly lower body mass index (BMI) and waist-hip ratio (WHR) as compared to those with preserved pancreatic function.

Since the presence of these autoantibodies may be influenced by disease progression, data were also analysed according to disease duration. There were 102 patients who were diagnosed within 5 years, 56 had been diabetic for 6 - 10 years, and the remaining 55 patients have had the disease for more than 10 years (Table III). IAA were consistently present in 45 - 53% of the patients in all the 3 groups. In contrast, the prevalence of GAD Ab was highest amongst the recent-onset group, but declined significantly (p=0.0001) to about 24 - 25% in those with long-standing diabetes. Whilst GAD Ab and especially IA2, were detected mostly in patients with inadequate post-glucagon C-peptide response, IAA was less specific, present even in patients whose pancreas were still preserved (Table IV).

Discussion

Antibody screening in the prediction and diagnosis of Type 1 diabetes mellitus has been found to be a useful procedure especially in the Caucasian populations[1,2]. Early detection of these antibodies before clinical onset may be an important step towards justifying for immunointervention strategies to prevent the development of Type 1 DM. The ICA, IAA and more recently, GAD Ab are recognised immune markers for Type 1 diabetes. However, this seems to be true only for the Caucasian subjects. In the Asian population, autoimmunity has been found not to be the major factor in the pathogenesis of Type 1 DM[16-18]. As was also

Table I
Frequency of GAD Ab, IA2, IAA and ICA in Malaysian Type 1 DM Patients

	GAD Ab	IA2	IAA	ICA
Number	72 (33.8%)	19 (8.9%)	101 (47.4%)	3 (1.4%)
Male/female	31/41	10/9	46/55	1/2
°C-peptide (pmol/L)				
Fasting	152 ± 19	103 ± 25	248 ± 30	512 ± 488
Post-glucagon	188 ± 27	140 ± 35	397 ± 51	982 ± 867

° *Mean ± SEM*

Table II
Characteristics of Type 1 DM Patients According to Autoimmune Status and Pancreatic Reserves

°Post-glucagon C-peptide	< 600 (n=172, 80.7%)		≥ 600 (n=41, 19.3%)	
Antibody status	**Positive (n=126)**	**Negative (n=46)**	**Positive (n=19)**	**Negative (n=22)**
Age at diagnosis (y)	13.4 ± 8.3	12.2 ± 8	19.4 ± 9.5	19.6 ± 7.6
/Disease duration (y)	7.5 ± 6.9	8.0 ± 6.4	6.8 ± 6.1	7.8 ± 7.3
BMI (kg/m^2)	20.3 ± 5.3	21.4 ± 3.8	24.4 ± 4.0	22.4 ± 3.2
WHR	0.81 ± 0.1	0.82 ± 0.06	0.87 ± 0.05	0.84 ± 0.06

° *Mean ± SEM*

Table III
Frequency of GAD Ab, IA2, IAA and ICA in Malaysian Type 1 DM Patients Analysed According to Disease Duration

Disease duration (y)	≤ 5 (n=102)	6 - 10 (n=56)	> 10 (n=55)
GAD Ab	[b]45 (44.1%)	14 (25.0%)	13 (23.6%)
IA2	[b]16 (12.8&)	2 (3.6%)	1 (1.8%)
IAA	47 (46.1%)	25 (44.6%)	29 (52.7%)
ICA	2 (2.0%)	1 (1.8%)	0 (0%)
[a]C-peptide (pmol/L)			
Fasting	261 ± 28	249 ± 44	225 ± 35
Post-glucagon	483 ± 49	366 ± 72	362 ± 63

[a] *Mean ± SEM*
[b] *p=0.0001 versus other long-standing diabetes groups (Fisher's exact test)*

Table IV
Frequency of GAD Ab, IA2 and IAA in Malaysian Type 1 DM Patients Analysed According to Disease Duration and Pancreatic Reserves

Disease Duration	≤ 5 years		6 - 10 years		>10 years	
Post-glucagon C-peptide (pmol/L)	< 600	> 600	< 600	> 600	< 600	> 600
GAD Ab	43/45 (95.6%)	2/45 (4%)	13/14 (92.9%)	1/14 (7.1%)	11/12 (91.7%)	1/12 (8.3%)
IA2	16/16 (100%)	0/16 (0%)	2/2 (100%)	0/2 (0%)	1/1 (100%)	0/1 (0%)
IAA	37/47 (78.7%)	10/47 (21.3%)	20/25 (80%)	5/25 (20%)	25/29 (86.2%)	4/29 (13.8%)

observed in this study, despite being clinically diagnosed as Type 1 DM, the prevalence of IAA and GAD Ab were only 47.4% and 33.8% respectively, while that of IA2 and ICA were lower, at 8.9% and 3% respectively. Although the number of seropositive patients could be increased to 145 (68%) when 2 or more antibodies were considered together, the cost and time required to test for these markers may outweigh their diagnostic usefulness. On the other hand, if the diagnosis of Type 1 and hence the need for insulin treatment was based on their C-peptide response to glucagon stimulation, then a total of 172 (80.2%) of the 213 patients would have been correctly classified and promptly treated, without the need to wait for the multiple analyses of the antibodies. Nevertheless, it is interesting to note that although the prevalence of GAD Ab was highest in the newly diagnosed patients (<5 years), unlike IA2 and ICA, the immune marker remained detectable in about 24 - 25% of those patients with long-standing disease, confirming its persistence[11,19] and in agreement with that observed in a group of Japanese Type 1 DM patients[20]. Insulin autoantibodies on the other hand, has been shown to be less specific, found not only in Type 1 DM but also in patients with other autoimmune disorders[21]. Based on the fact that over 92% of our patients who were positive to GAD Ab and/or IA2 were also found to have inadequate pancreatic reserve, it strongly suggests that these antibodies do play some roles in causing ß-cell damage in these subjects.

On the contrary, measurement of autoantibodies in

diabetic patients even in those who showed adequate post-glucagon C-peptide response may identify patients who belong to the LADA subset. As shown by Zimmet *et al.*[22], majority of the patients who presented with diabetes after the age of 30 years, and found to have GAD Ab, developed dependency on insulin for glycemic control, indicating a slow but progressive destruction of the pancreatic cells. Thus, detection of GAD Ab in adult-onset diabetes patients has been suggested to be useful as it would identify this group of patients at earliest possible stage and thus justifying early intervention therapy. However, in this study, only 4 (21%) of the 19 patients who could be categorised as 'LADA' were positive to GAD Ab. Our results were comparable to those reported for other Asian populations; GAD Ab frequency was only 25% in a cohort of Thai Type 2 patients with secondary sulfonylurea failure[23] and only 14% in a similar group of Japanese diabetics[24]. Thus, relying on the detection of GAD Ab may not be as effective and ideal strategy to identify LADA among the Asian diabetic subjects.

It has been proposed that prediction of Type 1 DM in the general population can be made possible and sufficiently sensitive by simultaneous screening for GAD Ab, IAA, IA2 and ICA[8,25-27]. Although the method will allow early detection and hence possible prevention, the cost of actually carrying it out would pose a major financial burden to most countries. More importantly, there are differences in the etiology of Type 1 between Caucasians and non-Caucasians[15,17,22]. As shown in this study, although a good percentage of our patients were positive for one or combination of the antibodies, there were still about 32% (68/213) who remained seronegative but yet presented with near or complete ß-cell destruction. Thus, there is a possibility that other, yet to be identified autoantigens are involved, which is perhaps more immunoreactive and specific to the Asian populations.

Acknowledgements

This study was supported by a research grant 06-02-02-0040 from the Ministry of Science, Technology and Environment, Malaysia. The authors wish to thank all the Medical Laboratory Technologists who were involved in this study, and the Director of Institute for Medical Research for permission to publish this study. We also would like to thank Novo Nordisk (Malaysia) Sdn Bhd for the supply of glucagon used for this study.

References

1. Palmer J-P Predicting IDDM; use of humoral immune markers. Diabetes Rev 1993; 1: 104-15.
2. Landin-Olsson M, Palmer J-P, Lernmark A, *et al.* Predictive value of islet cell and insulin autoantibodies for Type 1 (insulin-dependent) diabetes mellitus in a population-based study of newly-diagnosed diabetic and matched control children. Diabetologia 1992; 35: 1068-73.
3. Landin-Olsson M. Precision of the islet cell antibody assay depends on the pancreas. J Clin Lab Anal 1990; 4: 289-94.
4. Stumpo R-R, Llera A-S, Cardoso A-I, Poskus E. Solid versus liquid phase assays in detection of insulin antibodies. Influence of iodination site on labelled insulin binding. J Immunol Methods 1994; 169: 241-9.
5. Palmer J, Asplin C, Clemons P. Insulin autoantibodies in insulin-dependent diabetes before insulin treatment. Science 1983; 222: 1337-9.
6. Schmidli R-S, Colman P-G, Bonifacio E and participating laboratories. Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies. Diabetes 1995; 44: 636-40.
7. Falorni A, Ortqvist E, Persson B, Lernmark A. Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using 35S or 3H recombinant human ligands. J Immunol Meth 1995; 186: 89-99.

8. Hagopian W-A, Sanjeevi C-B, Kockum I, *et al.* Glutamate decarboxylase, insulin- and islet cell-antibodies and HLA-typing to detect diabetes in a general population-based study of Swedish children. J Clin Invest 1995; 95: 1505-11.

9. Kobayashi T, Itoh T, Kasaka K, Sato K, Tsuji K. Time course of islet cell antibodies and ß-cell function in non-insulin-dependent stage of Type 1 diabetes. Diabetes 1987; 36: 510-17.

10. Christie M, Delovitch T-L. Persistence of antibodies to a 64,000-Mr islet cell protein after onset of type 1 diabetes. Diabetes 1990; 39: 653-9.

11. Tuomilehto J, Zimmet P, Mackay I-R, *et al.* Antibodies to glutamic acid decarboxylase as predictors on insulin-dependent diabetes mellitus before clinical onset of disease. Lancet 1994; 343: 1383-5.

12. Tuomi T, Groop L-C, Zimmet P-Z, *et al.* Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. Diabetes 1993; 42: 359-62.

13. Niskanen L-K, Tinamaija T, Karjalainen J, Groop L-C, Uusituoa M-I-J. GAD antibodies in NIDDM. Ten-year follow-up from diagnosis. Diabetes Care 1995; 18: 1557-65.

14. Verge C-F, Gianani R, Kawasaki E, *et al.* Prediction of type 1 diabetes in first-degree relatives using combination of insulin, GAD, and ICA512bdc/1A-2 autoantibodies. Diabetes 1996; 45: 926-33.

15. Bingley P-J, Christie M-R, Bonifacio E, *et al.* Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. Diabetes 1994; 43: 1304-10.

16. Wan Nazaimoon W-M, Faridah I, Singaraveloo M, *et al.* Prevalence of glutamic acid decarboxylase antibodies amongst young Malaysian diabetics. Diab Res Clin Prac 1999; 43: 59-66.

17. Thai A-C, Ng W-Y, Loke K-Y, Lee W-R, Lui K-F, Cheah J-S. Anti-GAD antibodies in Chinese patients with youth and adult-onset IDDM and NIDDM. Diabetologia 1997; 40: 1425-30.

18. Park Y, Lee H, Koh C-S, *et al.* The low prevalence of immunogenetic markers in Korean adult-onset IDDM patients. Diab Care 1996;19: 241-5.

19. Savola K, Sabbah E, Kulmala P, Vähäsalo,Ilonen J, Knip M. Autoantibodies associated with Type ! diabetes mellitus persist after diagnosis in children. Diabetologia 1998; 41: 1293-7.

20. Imagawa A, Itoh N, Hanafusa T, *et al.* High prevalence of antibodies to glutamic acid decarboxylase in comparison to islet cell antibodies in patients with long-standing insulin-dependent diabetes mellitus. Res Commun Mol Pathol Pharmacol. 1996; 92: 43-52.

21. Wilkin T, Hoskins P-J, Armitage M, *et al.* Value of insulin autoantibodies as serum markers for insulin-dependnet diabetes mellitus. Lancet 1985; 1: 480-1.

22. Zimmet P-Z, Rowley M-J, Mackay I-R, *et al.* The ethnic distribution of antibodies to glutamic acid decarboxylase: presence and levels of insulin-dependent diabetes mellitus in Europid and Asian subjects. J Diab Compli 1993; 7: 1-7.

23. Rattanasarn C, Diosado M-A, Soonthornpun S. Glutamic acid decarboxylase antibodies in non-insulin-dependent diabetes patients with secondary sulfonylurea failure in Thailand. Diab Res Clin Prac 1997; 37: 193-7.

24. Fukui M, Nakano H, Shigeta H, *et al.* Antibodies to glutamic acid decarboxylase in Japanese diabetic patients with secondary failure of oral hypoglycaemic therapy. Diab Med 1997; 14: 148-52.

25. Dittler J, Seidel D, Schenker M, Ziegler A-G. GADIA2-combi determination as first-line screening for improved prediction of type 1 diabetes in relatives. Diabetes 1998; 47: 592-7.

26. Bingley P-J, Bonifacio E, Williams A-J, Genovese S, Bottazzo G-F, Gale E-A. Prediction of IDDM in the general population; strategies based on combinations of autoantibody markers. Diabetes 1997; 46: 1701-10.

27. Ongagna J-C, Levy Marchal C. Sensitivity at diagnosis of combined beta-cell autoantibodies in insulin-dependent diabetic children. French Registry of IDDM in children Study Group. Diabetes Metab. 1997; 23: 155-60.