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Combined screening for autoantibodies to IA-2 and antibodies to glutamic acid decarboxylase in first degree relatives of patients with IDDM

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Summary To determine the value of antibodies to the intracytoplasmic domain of the tyrosine phosphatase IA-2 (anti-IA-2 ic) and glutamic acid decarboxylase (GADA) for identification of subjects at risk for insulin-dependent diabetes mellitus (IDDM) we investigated 1238 first degree relatives of patients with IDDM for the presence of anti-IA-2 ic and GADA and compared the results with cytoplasmic islet cell antibodies (ICA). Anti-IA-2 ic were observed in 54 (4.4 %) first degree relatives, in 51 of 86 (59.3 %) ICA positive relatives and in 3 of 4 individuals who developed overt IDDM within a follow-up period of 1 to 28 months. GADA were found in 78 of 1238 (6.3 %) first degree relatives. They were detected in 22 of 35 (62.9 %) sera with ICA alone and in 1 of 3 subjects with anti-IA-2 ic in the absence of ICA. Of the 1238 subjects 37 (3.0 %) sera were positive for all three antibodies. Both anti-IA-2 ic and GADA were positively correlated with high levels of ICA. Anti-IA-2 ic and GADA were detected in 39.1 and 47.8 % of subjects with ICA of less than 20 Juvenile Diabetes Foundation units (JDF-U) but in 66.7 and 76.2 % of individuals with ICA of 20 JDF-U or more, respectively ($p < 0.05$). The levels of ICA and GADA in first degree relatives with at least one additional marker were significantly higher than in subjects with ICA alone ($p < 0.005$) or GADA alone ($p < 0.03$). The combination of anti-IA-2 ic and GADA identified 84.9 % of all ICA positive subjects and 93.7 % of individuals with high level ICA (≥ 20 IDF-U). All 4 individuals who progressed to IDDM had either IA-2 ic or GADA. Our data indicate that primary screening for anti-IA-2 ic and GADA provides a powerful approach with which to identify subjects at risk for IDDM in large-scale population studies which may represent the basis for the design of new intervention strategies. [Diabetologia (1996) 39: 1351–1356]

Keywords Insulin-dependent diabetes mellitus, antibodies to tyrosine phosphatase IA-2, GAD antibodies, islet cell antibodies, prediction.

Insulin-dependent diabetes mellitus (IDDM) is the result of a chronic autoimmune process characterised by the appearance of several islet cell specific autoantibodies. Various studies have shown that the presence of diabetes–associated autoantibodies in non-diabetic individuals confers increased risk for the future development of IDDM. At present, screening for cytoplasmic islet cell antibodies (ICA) represents the most widely established procedure with which to estimate the risk for IDDM. Depending on the levels of ICA a positive ICA test in first degree relatives of IDDM patients indicates a risk in the range of 34 to 100 % within 10 years [1–4]. The usefulness of ICA for routine screening of susceptible individuals or the general population is, however, limited by the cumbersome nature of the ICA assay and problems

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Abbreviations: ICA, Islet cell antibodies, IDDM, insulin-dependent diabetes mellitus; IAA, insulin autoantibodies; GADA, autoantibodies to glutamic acid decarboxylase; 40 kDa-ab, autoantibodies to 40 kDa antigen; 37 kDa-ab, autoantibodies to 37 kDa, antigen; IA-2ic, tyrosine phosphatase IA-2; JDF-U, Juvenile Diabetes Foundation units.

* See acknowledgements

with the consistency of the test [5, 6]. Therefore, several studies have analysed the value of other antibody markers for prediction of IDDM.

It has been shown that the prediction of IDDM can be considerably improved by the addition of insulin autoantibodies (IAA), autoantibodies to glutamic acid decarboxylase (GADA) and autoantibodies to 37 kDa (37 kDa-ab) and 40 kDa (40 kDa-ab) antigens in ICA positive individuals [7–12]. Primary screening for either IAA or GADA alone, however, has proved to be less specific than ICA testing [7, 13–15]. In contrast, 37 kDa-ab and 40 kDa-ab have been reported to be strongly associated with rapid progression to IDDM, but like other markers these antibodies are unable to identify all individuals who later develop IDDM [4, 11]. Due to the labour intensive test procedure using radiolabelled islet cells, it has been difficult to screen large numbers for 37 kDa-ab/40 kDa-ab so far [16]. Recently, Christie and co-workers [16, 17] identified the 40 kDa antigen as the intracytoplasmic domain of the tyrosine phosphatase IA-2 (IA-2ic) [17, 18]. With the cloning of the human IA-2 cDNA, population-based screening for autoantibodies to IA-2 is now possible. In this study we describe a radiobinding assay which allows quantitative measurement of autoantibodies to IA-2ic (anti-IA-2ic) on a large scale. We also show population-based data on the prevalence of IA-2 antibodies and demonstrate that combined screening for anti-IA-2ic and GADA identify almost all subjects with high levels of ICA in first degree relatives of patients with IDDM. These data suggest that screening for antibodies to IA-2 in combination with GADA may represent a powerful strategy for routine screening to identify subjects at increased risk for IDDM.

Subjects and methods

Patients. Sera were obtained from the first sample of 1238 non-diabetic first degree relatives of IDDM patients (663 females, 575 males; age 0.1 and 57 years, mean age 12.7 ± 11.6 years), who were recruited by the screening programme of the Deutsche Nikotinamid Interventions-Studie (DENIS). Sera of probands participating in the DENIS Study were collected before the start of study treatment. Sera from 100 healthy individuals (mean age 16.5 years, range 7–28 years) without a family history of IDDM were used as controls. Informed consent was obtained from the subjects or their parents. The study was approved by the ethical committees of the centres participating in the study.

Detection of autoantibodies to human recombinant GAD 65 and IA-2. Autoantibodies to the intracytoplasmic domain of IA-2 (anti-IA-2ic) and GADA were determined using radiolabelled human recombinant antigens in a 96-well assay format. Recombinant autoantigens were produced by coupled in vitro transcription and translation (Promega, Madison, Wis., USA) of human IA-2ic cDNA, coding for IA-2ic (amino acids 603–980) [17] and human GAD 65 cDNA (a kind gift of Å. Lernmark, Seattle, USA) [19], respectively.

Plasmid cDNA (1 μg) coding either for GAD 65 or IA-2ic was incubated with the reticulocyte lysate system and ^{35}S methionine (10 mCi/ml, > 1000 Ci/mmol; Amersham Ltd., Amersham, Bucks., UK) according to the manufacturer's instructions. Incorporation of radioactivity in recombinant proteins was monitored by precipitation with trichloroacetic acid. We incubated 10,000 cpm of in vitro synthesized IA-2ic, or 20,000 cpm of GAD 65 with 5 μl serum diluted in 50 μl buffer A (mmol/l 20 Tris, 150 NaCl, pH 7.4 with 0.1 % bovine serum albumin, 5 methionine, 5 benzamidine, 2 PMSF, 2 EDTA, 0.1 % trasylol, 0.5 % Triton X 100) in 96-well microtitre plates (Greiner, Nürtingen, Germany). After overnight incubation on a rotating platform 20 μl Protein A Sepharose (50 % v/v) was added for 2 h followed by the transfer of the probes into prewashed 96-well filtration plates (Multiscreen BV 1.2 μm; Millipore, Bedford, Mass., USA). Plates were extensively washed in buffer A (15 × 150 μl) and precipitates were punched out into 5 ml scintillation vials (Multiple 8-punch system; Millipore) to count bound proteins in a liquid scintillation counter. Alternatively, immunoprecipitates were subjected to SDS-PAGE and analysed by autoradiography. In each experiment the same positive and negative standard sera were included in duplicates. Antibody levels were expressed as arbitrary units (AU) calculated as follows: U = (cpm[test serum] – cpm[negative standard serum]) / (cpm[positive standard serum] – cpm(negative standard serum]) × 100. Intra-assay and inter-assay coefficients of variation of the IA-2ic antibody assay were 7.7 % ($n = 9$) and 10.9 % ($n = 9$), respectively. To achieve high specificity of antibody detection the cut off for antibody positivity was set at mean + 4 SD of antibody levels in 100 normal control sera (7 GAD-U and 3 IA-2-U). In the Second GAD Antibody Proficiency Program our GADA assay achieved 100 % sensitivity and 100 % specificity.

Detection of cytoplasmic ICA. Islet cell antibodies were detected by the indirect immunofluorescence test on unfixed cryostat sections of human pancreas from an organ donor with blood group 0 as described [20]. Determinations were carried out on the same tissue and the results were expressed in Juvenile Diabetes Foundation JDF units according to the workshop protocol for the standardisation of ICA. The detection limit of the assay in our laboratory was 3 JDF-U. In the 10 th International Diabetes Workshop ICA Proficiency Program our laboratory achieved values of 90 % for sensitivity and 100 % for specificity (Laboratory identification No 298).

Statistical analysis

The significance of differences between observations was tested using the Wilcoxon test, the chi-square test with Yates' correction, Fisher's exact test, or Mann-Whitney test where appropriate. The significance of correlations between antibodies was tested by Spearman rank correlation analysis.

Results

Prevalence of autoantibodies. Among 1238 non-diabetic first degree relatives of patients with IDDM 54 (4.4 %) were positive for anti-IA-2ic (mean age 10.5 ± 9.9 years, 26 females, 28 males), 78 (6.3 %) had GADA (mean age 11.0 ± 8.9 years, 35 females,

Table 1. Antibodies to IA-2 ic (anti-IA-2 ic), antibodies to glutamic acid decarboxylase (GADA) and cytoplasmic islet cell antibodies (ICA) in first degree relatives of patients with IDDM stratified by age

Subjects Age (years)	Number	Prevalence of autoantibodies		
		Anti-IA-2 ic	GADA	ICA
0–6	443	19 (4.3)	28 (6.3)	25 (5.6)
7–12	494	27 (5.5)	32 (6.5)	44 (8.9)
13–18	99	4 (4.0)	11 (11.1)	9 (9.1)
19–30	43	2 (4.7)	2 (4.7)	2 (4.7)
> 31	159	2 (1.3)	5 (3.1)	6 (3.8)
Total	1238	54 (4.4)	78 (6.3)	86 (6.9)

Data are *n* (%)

43 males) and 86 (6.9%) were ICA positive (mean age 11.4 ± 8.8 years, 39 females, 47 males). Only 1 of 100 (1.0%) normal control subjects had GADA (range 0–9.0 GAD-U), and none of 100 individuals were positive for anti-IA-2 ic (range 0–2.8 IA-2 ic-U) or ICA ($p < 0.05$) (Table 1). Of these subjects 33 (2.7%) were positive for only one test, while 37 (3.0%) subjects had two markers and 37 (3.0%) subjects were found positive for all three markers, i.e. ICA, anti-IA-2 ic and GADA. There was no significant association of any antibody with age or gender.

Association between IA-2 ic antibodies, GADA and ICA. The distribution of the three antibody specificities within the study population is illustrated in Figure 1. Both anti-IA-2 ic as well as GADA were positively associated with the presence of ICA. IA-2 ic antibodies were detected in 51 (59.3%) and GADA in 59 (68.6%) of 86 ICA positive subjects, respectively ($p < 0.0001$) (Fig. 1). Significantly higher frequencies of anti-IA-2 ic and GADA were found in subjects with ICA levels of 20 JDF-U or more (42/63 [66.7%] and 48/63 [76.2%], $p < 0.05$) or with ICA levels of 40 JDF-U or more (35/50 [70.0%] and 40/50 [80.0%], $p < 0.02$) compared to individuals with ICA less than 20 JDF-U (9/23 [39.1%] positive for anti-IA-2 ic and 11/23 [47.8%] GADA positive). Combined screening for the presence of either anti-IA-2 ic or GADA identified 84.9% of all ICA positive relatives. Most strikingly, anti-IA-2 ic or GADA were present in 93.7% (59/63) and 96.0% (48/50) subjects with ICA levels 20 JDF-U or more and 40 JDF-U or more, respectively (Fig. 2). As illustrated in Figure 2 only 3 of 1152 (0.26%) ICA negative relatives had anti-IA-2 ic. In one of these three cases high levels of GADA (80.2 GAD-U) were also detected. Out of 35 ICA positive anti-IA-2 ic negative sera 22 (62.9%) were found to be positive for additional GADA. GADA in the absence of ICA and anti-IA-2 ic were observed in 18 of 78 (23.1%, 1.5% of the total population) GADA positive subjects, which is similar to the prevalence of GADA observed in normal control subjects (1.0%).

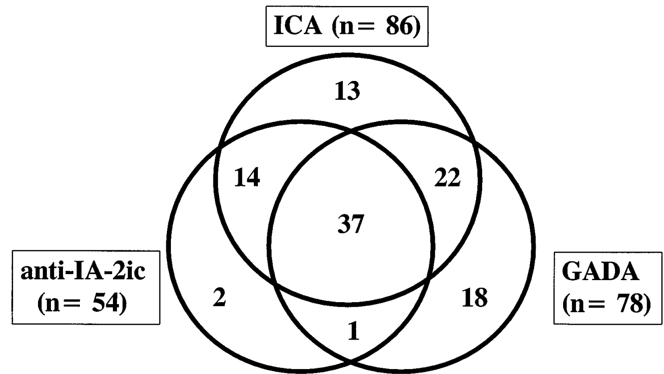

Fig. 1. Combinations of autoantibodies in 107 antibody positive first degree relatives of patients with IDDM. Antibodies to IA-2 ic (anti-IA-2 ic), antibodies to glutamic acid decarboxylase (GADA) and cytoplasmic islet cell antibodies (ICA)

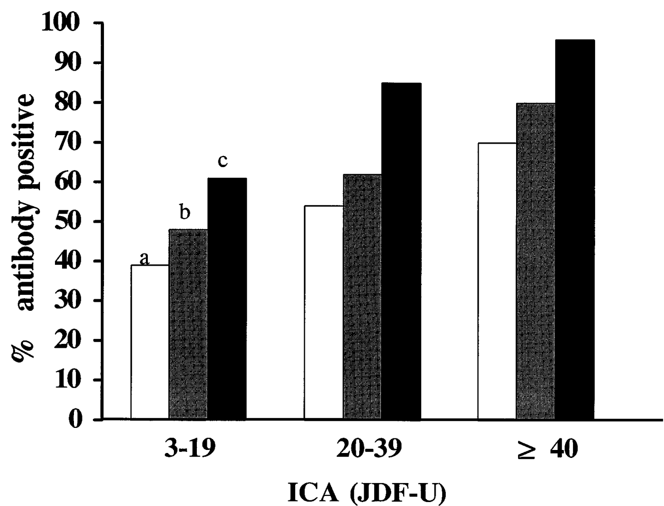

Fig. 2. Association of antibodies to IA-2 ic (anti-IA-2 ic) and antibodies to glutamic acid decarboxylase (GADA) grouped according to levels of islet cell antibodies (ICA). Anti-IA-2 ic positive (□); GADA positive (■); anti-IA-2 ic positive and/or GADA positive (▒). a $p < 0.02$; b $p < 0.01$; c $p < 0.0005$ vs subjects with ICA ≥ 40 JDF-U

In the 13 relatives who had ICA alone (1.1% of the total population), ICA levels (15.8 ± 11.5 JDF-U) were significantly lower than in sera with one (30.2 ± 16.1 JDF-U, $p < 0.005$) or two additional markers (33.9 ± 14.6 JDF-U, $p < 0.0005$). A similar result was observed when GADA levels were compared between single GADA positive sera (31.3 ± 16.8 GAD-U) and sera with two or three autoantibodies (56.3 ± 28.2 GAD-U) ($p < 0.03$). Analysis of autoantibody levels in individual sera revealed a significant correlation between ICA and anti-IA-2 ic ($r = 0.59$, 95% confidence interval: 0.44–0.70, $p < 0.001$) as well as ICA and GADA ($r = 0.21$, 95% confidence interval: 0.01–0.39, $p < 0.05$). There was no correlation between the levels of anti-IA-2 ic and GADA (Fig. 3).

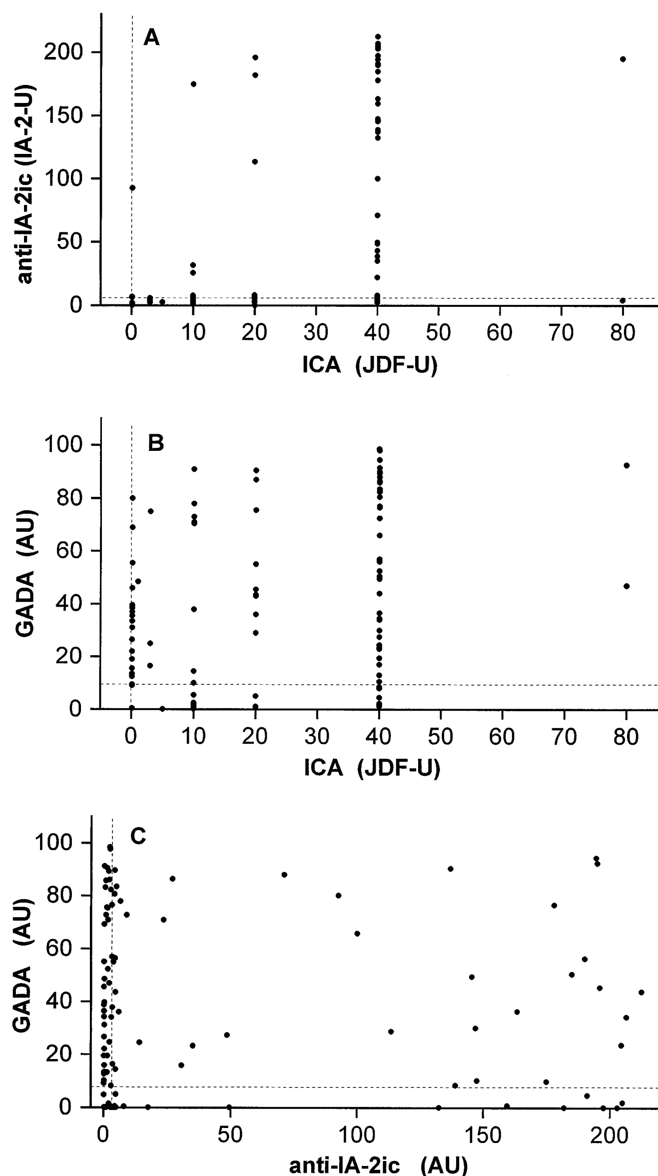

Fig. 3. A–C Relationship between autoantibody levels in first degree relatives of patients with IDDM. Levels of islet cell antibodies (ICA) were compared with levels of antibodies to IA-2ic (anti-IA-2ic) **(A)** and levels of antibodies to glutamic acid decarboxylase (GADA) **(B)**. The levels of anti-IA-2ic were correlated with levels of GADA **(C)**. Anti-IA-2ic and GADA levels are expressed in arbitrary units (AU) and ICA are given in JDF-U

Follow-up investigation. During a mean follow-up of 23 months (range 1–31 months) 4 out of 107 autoantibody positive subjects (3 females, 1 male; mean age 9.3 ± 3.2, range 5.8–13.5 years) but none of the antibody negative individuals developed overt IDDM. At initial screening (1, 14, 16 and 28 months before the development of IDDM) all prediabetic subjects were positive for ICA (40 JDF-U). In one child high levels of anti-IA-2ic (163 IA-2-U) and GADA (36 GAD-U) were present; two children had high levels of anti-IA-2ic (173 and 160 IA-2-U) but were negative for GADA (1.9 and 0.8 GAD-U) and one child was GADA positive (13 GAD-U) and anti-IA-2ic negative (1.4 IA-2U). All 4 prediabetic subjects were positive for either anti-IA-2ic or GADA.

Discussion

In this study we assessed the value of antibodies to human recombinant IA-2 and GAD to identify subjects at increased risk for IDDM in a large population of first degree relatives of patients with IDDM. Since the short follow-up period does not allow accurate analysis of the predictive value of the antibodies, we compared anti-IA-2ic and GADA with ICA which is the best validated serological marker found so far to estimate the risk for IDDM [1–4]. We report here that combined screening for anti-IA-2ic and GADA detected more than 93 % of subjects who possess a high risk for IDDM on the basis of high ICA titres, and all 4 individuals who subsequently developed overt IDDM during the follow-up period were positive for either anti-IA-2ic or GADA. The use of 96-well radiobinding techniques to measure these antibodies allows rapid and quantitative screening for autoantibodies on a large scale, overcoming some of the limitations of the ICA test [5, 6].

Anti-IA-2ic were found to be present in 4.4 % of the large cohort of 1238 first degree relatives which is a prevalence comparable with the frequencies of ICA or IAA reported from several family studies and is similar to the estimated cumulative incidence of IDDM in first degree relatives [1–3, 7]. We observed a strong association of anti-IA-2ic with the presence of ICA confirming previous reports on antibodies to the 37 kDa/40kDa antigens [4, 10, 12, 21]. Thus, ICA were present in all but three sera with anti-IA-2ic. Conversely, anti-IA-2ic were found in 59.3 % of subjects with detectable ICA (≥ 3 JDF-U). The prevalence of anti-IA-2ic in ICA positive subjects was higher than in previous studies where antibodies to the 37 kDa/40kDa antigens were detected in only 17 % of ICA positive first degree relatives [4, 21]. These studies are not strictly comparable because of differences in protocols for measurement of autoantibodies. The use of recombinant antigen in radioligand binding assays may increase the sensitivity of detection, as described for GADA [22–24]. Anti-IA-2ic were detected in 3 of the 4 subjects who progressed to IDDM during a mean follow up period of 23 months. The high prevalence of anti-IA-2ic in prediabetic and ICA positive relatives is consistent with previous reports which have described antibodies to the 40 kDa antigen in a high proportion of prediabetic relatives and ICA positive patients with recent-onset IDDM [4, 10, 11]. The segregation of anti-IA-2ic positive relatives in a subgroup of ICA positive subjects with high risk for IDDM indicates

that these antibodies are valuable tools in the prediction of IDDM.

Although a large proportion of ICA positive subjects are identified by anti-IA-2 ic, primary screening for anti-IA-2 ic alone does not detect all the individuals at risk for IDDM. To improve sensitivity we combined anti-IA-2 ic with GADA which can be easily detected using recombinant antigen in the same assay format [25]. As for anti-IA-2 ic, GADA were positively associated with the presence of ICA, and in particular with high levels of ICA. These findings are in agreement with previous observations on a preferential occurrence of GADA in ICA positive prediabetic subjects and patients with IDDM [22, 25–27]. Positivity for GADA alone, in the absence of other antibody markers, which is known to be correlated with a low risk for IDDM [13, 14], was observed in only 1.5 % of the total population. Although screening for GADA may identify individuals who are unlikely to develop IDDM, these are rare. It is important to note, however, that GADA identified 62.9 % of ICA positive subjects who were negative for anti-IA-2 ic. Risk estimation in several family studies revealed that the additional presence of GADA in sera of ICA positive first degree relatives indicates an increased risk for IDDM. Inclusion of GADA may thus complement anti-IA-2 ic in screening programmes and may increase sensitivity for prediction of IDDM [4, 12]. Despite a high correlation between GADA or anti-IA-2 with ICA 13 (1.05 % of the study population) individuals were found exclusively ICA positive. Of those 13 subjects 9 (69.2 %) had ICA levels of less than 10 JDF-U indicating that only a small number of subjects who may progress to overt IDDM might be missed by the combined screening of anti-IA-2 ic and GADA.

In conclusion, the use of human recombinant autoantigens IA-2 and GAD made it possible to develop radiobinding assays which allow rapid and sensitive detection of two major targets of humoral autoimmunity in IDDM. Our findings indicate that the combination of anti-IA-2 ic and GADA can accurately identify subjects at risk for IDDM identified on the basis of ICA positivity. The present approach offers an alternative strategy which might possess the power to replace ICA for primary screening in large-scale population studies. At present the use of both anti-IA-2 ic and GADA is limited by the still unknown predictive values of both markers which need to be validated in prospective family studies and the general population. Accurate risk estimation, however, can be achieved by a two-step procedure whereby initial combined screening for GADA and anti-IA-2 ic is followed by scoring of ICA in those subjects positive for one marker. This strategy may considerably facilitate population-based screening programmes to identify subjects for intervention trials to prevent the development of IDDM.

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