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Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity

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Summary Identification of islet autoantigens offers the possibility that antibody tests other than islet cell antibodies may be used for assessing risk of insulin-dependent diabetes mellitus (IDDM). The aim of this study was to determine the combination of islet autoantibody markers that could identify most future cases of IDDM. Islet cell antibodies, antibodies to glutamic acid decarboxylase $(GAD)_{65}$, 37,000/40,000 M_r islet tryptic fragments, carboxypeptidase-H, and islet cell autoantigen (ICA)69 were measured in sera from 100 newly-diagnosed IDDM patients, 27 individuals prior to onset of IDDM, and 83 control subjects. Islet cell antibodies were detected in 88 % of IDDM patients and 81 % with pre-IDDM, GAD_{65} antibodies in 70 % of IDDM patients and 89 % with pre-IDDM, and antibodies to 37,000/40,000 M_r islet tryptic fragments in 54 % of IDDM patients and in 48 % with pre-IDDM. The latter were found only in conjunction with islet cell antibodies and were more frequent in young onset cases. All 20 IDDM patients and the 3 pre-IDDM subjects who had islet cell antibodies without GAD_{65} antibodies had antibodies to 37,000/40,000 M_r islet tryptic fragments, and all but one had disease onset before age 15 years. No sera strongly immunoprecipitated in vitro translated ICA69 or carboxypeptidase-H; 4 % of patients had anti-ICA69 and 11 % anti-carboxypeptidase-H levels above those of the control subjects. The findings suggest that none of the single antibody specificities are as sensitive as islet cell antibodies, but that a combination of GAD_{65} antibodies and antibodies to 37,000/40,000 M_r islet tryptic fragments has the potential to identify more than 90 % of future cases of IDDM. Such a strategy could eventually replace islet cell antibodies in population screening for IDDM risk assessment. [Diabetologia (1995) 38: 816–822]

Key words Islet cell antibodies, glutamic acid decarboxylase$_{65}$ antibodies, islet autoantigens, insulin-dependent diabetes mellitus prediction, carboxypeptidase-H, ICA69.

Treatments that may delay or prevent the onset of insulin-dependent diabetes (IDDM) are already being tested in large-scale trials in family members of children with the disease [1]. Although current risk assessment strategies are based on prospective family studies [2–4], only around 10 % of patients have a first-degree family history of IDDM. If preventive therapies are to have a real impact on the frequency of disease, they will eventually have to be evaluated and applied in the population as a whole.

Islet cell antibodies (ICA) are the most widely used antibody marker in risk assessment [5]. Over 80 % of first-degree relatives who develop IDDM have detectable ICA, and unaffected relatives with ICA are at greatly increased risk of developing the disease [2–4]. The technical limitation of ICA measurements is that they rely on semi-quantitative indi-

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Abbreviations: CL, Confidence limits; CPH, carboxypeptidase-H; GAD, glutamic acid decarboxylase; GADA, glutamic acid decarboxylase$_{65}$ antibodies; IAA, insulin autoantibodies; ICA, islet cell antibodies; IDDM, insulin-dependent diabetes mellitus; JDF units, Juvenile Foundation Diabetes units.

rect immunofluorescent assays [6] which, despite much improvement [7], are difficult to standardize [8, 9]. Antibodies to the islet autoantigen glutamic acid decarboxylase (GAD) are also detected prior to onset of IDDM [10–14]. The recent development of GAD antibody (GADA) assays [15, 16] which use small volumes of serum and detect antibodies in over 70 % of IDDM patients, now offer the possibility to screen large sample numbers. Similar methods could potentially be applied to other proposed antibody markers [17] and it may ultimately be possible to develop a single assay measuring antibodies to several islet antigens, thus replacing ICA testing.

We have measured antibodies to GAD, 37,000/40,000 M_r islet tryptic fragments (anti-37 K) [18], carboxypeptidase-H (CPH) [19], and ICA69 [20], in 100 consecutive patients with newly-diagnosed IDDM, in 83 control subjects and in samples taken prior to onset of IDDM from 27 individuals in the Barts-Windsor [2] and Barts-Oxford family studies [13]. The aim of the study was to select the best combination of antibody markers for use in a screening test for IDDM risk assessment and to evaluate these in comparison with ICA.

Subjects, materials and methods

Subjects

Patients with IDDM. Serum samples were obtained at onset of disease from 100 consecutive patients with IDDM diagnosed at the San Raffaele Hospital, Milan, Italy, during 1990 and 1991. IDDM was defined on the basis of a clinical diagnosis. All patients started insulin at the time of diagnosis and remained insulin-dependent thereafter. Patients had a median age of 11 years (range 1 to 40 years). Sixty-nine were diagnosed before age 15 years, and 53 were male.

Pre-IDDM. Serum taken prior to diagnosis was available in 27 of the 29 family members who have developed IDDM whilst under follow-up in the Barts-Windsor [2] and Barts-Oxford family studies in the UK [13]. They were all included irrespective of ICA status. The first available serum sample from each individual was tested. Six parents and 21 siblings of the diabetic proband were studied. The median age at sample collection was 17 years (range 2 to 55 years) and at diagnosis of IDDM was 20 years (range 3 to 57 years). The median time to diagnosis was 2.7 years (range 0.2 to 8.1 years).

Control subjects. Samples were collected from 83 normal children and blood donors in the Milan area. They had a median age of 12 years (range 1 to 40 years), 37 were male.

Recombinant autoantigens

Poly-A RNA was isolated from 20,000 purified human islets using Dynabeads Oligo dT(25) (Dynal, Oslo, Norway) [21]. Reverse transcription of 3 µl of the poly-A was performed using a mixture of random hexamers (Perkin Elmer Cetus, Norwalk, Conn., USA) to obtain cDNA [22]. The full length coding sequences of GAD_{65} and ICA69 were obtained after polymerase chain reaction amplification [23] of cDNA. The amplified products were ligated into the plasmid vector pCRII (Invitrogen, San Diego, Calif., USA), and then subcloned into the ECoR1 (GAD_{65}) or BamH1 (ICA69) cloning sites of the pGEM 3 vector (Promega, Madison, Wis., USA) under the control of the SP6 promotor for in vitro transcription and expression [24]. Recombinant rat CPH cDNA, cloned into the pSP64 vector (Promega) under the control of the SP6 promotor, was kindly provided by Dr. J. Hutton, Addenbrooke's Hospital, Cambridge, UK.

Radiolabelled recombinant proteins were obtained from in vitro coupled transcription and translation of 1 µg of the appropriate plasmid by SP6 RNA polymerase and rabbit reticulocyte lysate (Promega) in the presence of 40 µCi ^{35}S-methionine (Amersham International, Amersham Bucks., UK). Unincorporated label was removed by gel chromatography on a NAP 5 column (Pharmacia, Uppsala, Sweden).

Autoantibody measurements

GADA. Antibodies to in vitro translated GAD_{65} were measured using a method similar to that previously described [16]. A total of 15,000 cpm of labelled GAD_{65} protein diluted in 50 mmol/l Tris-HCl pH 7.2, 150 mmol/l NaCl, and 1 % Tween 20 (TBST) was added to 2 µl of serum to a final volume of 50 µl in 96-deep well microtitre plates and incubated overnight on ice. Immunocomplexes were isolated on 1 mg protein A Sepharose (Pharmacia), pre-swelled and resuspended in 50 µl of TBST, followed by incubation for 1 h at 4 °C with shaking. The immunocomplexes bound to protein A were washed once with 750 µl of cold TBST and transferred to a 96-well filtration system (Millipore, Bedford, Mass., USA) with a 0.45-µm filter at the bottom of the wells. The unit was placed on a vacuum device and after 10 washes, each of 150 µl of TBST, the bottom of each well was punched into a scintillation vial containing 2.5 ml of scintillation fluid (Ultimagold, Packard, Groningen, The Netherlands), and cpm measured in a scintillation counter (Kontron Instruments, Montigny le Bretonnieux, France). All samples were tested in duplicate. Results are expressed as mean cpm. The interassay coefficient of variation on 10 assays was less than 15 % for control samples with mean cpms of 300 and 600. This assay detected none of 32 control samples and 32 of 39 (82 %) IDDM samples in the 2nd International GAD antibody workshop.

Anti-carboxypeptidase-H and anti-ICA69. Antibodies to in vitro translated CPH and ICA69 were measured as previously described for anti-ICA69 [24]. A total of 17,000 cpm of labelled protein diluted in TBST was added to 4 µl of serum in a final volume of 50 µl in 96-deep-well microtitre plates and incubated overnight on ice. Immunocomplexes were isolated on 2 mg protein A Sepharose (Pharmacia), pre-swelled and resuspended in 50 µl of TBST, followed by incubation for 1 h at 4 °C with shaking. For anti-CPH, immunocomplexes bound to protein A were washed eight times, each with 750 µl of cold TBST, transferred to scintillation vials containing 2.5 ml of scintillation fluid, and the cpm measured. For anti-ICA69, immunocomplexes bound to protein A were washed twice, each with 750 µl of cold TBST and transferred to a 96-well filtration system, washed and counted as for GADA. Rabbit polyclonal anti-CPH provided by Dr. J. Hutton and a rabbit polyclonal antiserum raised against purified recombinant ICA69 protein (Primm, Milan, Italy) strongly immunoprecipitated the in vitro translated proteins (Fig. 1).

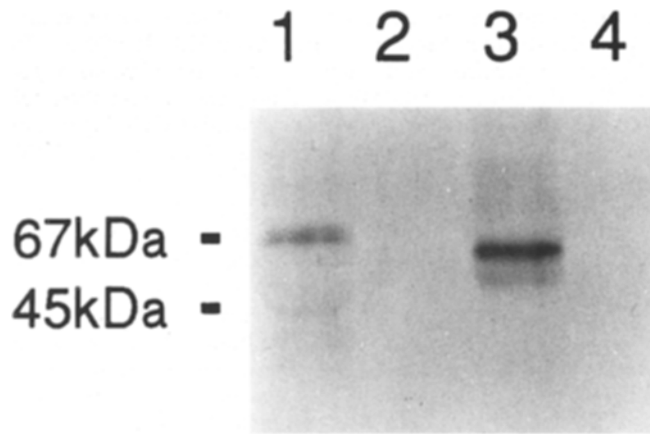

Fig. 1. Immunoprecipitation of in vitro translated, ICA69 (lanes 1 and 2) and CPH (lanes 3 and 4). ^{35}S methionine labelled protein was immunoprecipitated with 0.2 µl rabbit anti-ICA69 (lane 1), 0.2 µl rabbit anti-CPH (lane 3), and 2 µl preimmune rabbit sera (lanes 2 and 4), and run on 10 % polyacrylamide gel electrophoresis under denaturing conditions

Dilutions of these antisera were used as controls in each assay.

Anti-37 K. Rat insulinoma cell line (RIN 5AH) was maintained in tissue culture in RPMI 1640 medium containing 10 % fetal bovine serum (Sigma, St. Louis, Mo., USA). Subconfluent RIN cells in a 162-cm^2 tissue culture flask (approximately 1×10^8 cells) were radiolabelled in methionine-free RPMI 1640 medium (Sigma) with 1 mCi of [^{35}S] in vitro cell labelling mix (Promix – Amersham International) for 5 h at 37 °C; the cell pellet was then frozen at –80 °C until processed. For cell lysis, 400 µl of 10 mmol/l Hepes (pH 7.4), 150 mmol/l NaCl, 0.1 % (weight/volume) aprotinin, and 2 % Triton X-114 were added to a pellet of radiolabelled insulinoma cells followed by 2 h incubation at 4 °C with agitation. The homogenate was centrifuged at 10,000 *g* for 15 min at 4 °C, and the supernatant collected and incubated at 30 °C for phase separation. Triton X-114 detergent phase was precleared with 50 µl of normal human serum for 18 h at 4 °C followed by binding to 100 µl of packed protein A Sepharose (Sigma) for 30 min at 4 °C. Aliquots of 20 µl extract containing 4×10^6 cpm of radiolabelled protein were incubated with 5 µl of test serum for 5 h at 4 °C. Immune complexes were isolated on 20 µl of packed protein A Sepharose, washed three times with 1 ml 20 mmol/l Hepes (pH 7.4), 500 mmol/l NaCl, and once with 1 ml of water. They were then incubated with 50 µl of 0.1 mg/ml trypsin in 10 mmol/l Hepes (pH 7.4), and 150 mmol/l NaCl for 20 min at 4 °C, washed with 1 ml of water before processing for SDS-10 % polyacrylamide gel electrophoresis and autoradiography. Serum samples were regarded as anti-37 K positive if 37kDa and/or 40kDa polypeptide bands were detected on the autoradiogram.

Islet cell antibodies (ICA). These were measured in undiluted sera by indirect immunofluorescence on 4-µm cryostat sections of blood group O human pancreas as previously described [2]. Positive samples were titrated to end point in doubling dilutions in 10 mmol/l phosphate-buffered saline (pH 7.2). Local (Milan) standard sera calibrated to 2.5, 5, 10, 20, 40 and 80 Juvenile Diabetes Foundation (JDF) units were included in each assay. End-point titres of test samples were converted to JDF units by comparison with a standard curve of $\log_2$ JDF units vs $\log_2$ of end-point titre of the standard sera. The threshold of ICA detection was 2 JDF units.

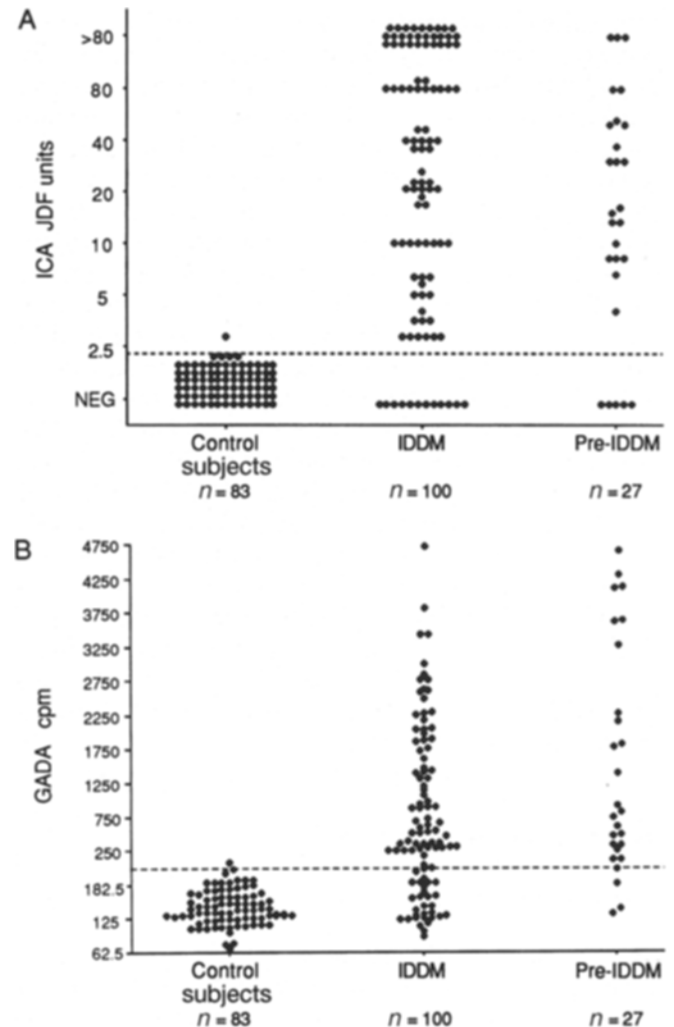

Fig. 2. (**A**) ICA and (**B**) GADA distribution in study groups. The upper first percentile of control subjects is shown by the broken lines

Insulin autoantibodies (IAA). These were measured on samples in which ICA were not detected using a radio-binding assay as previously described [25].

Statistical analysis

The threshold for positivity was selected as the upper first percentile of control results in each assay. All assays will have the same specificity (99 %) allowing comparison of sensitivities between them. All comparisons were made using the chi-square test with Yates' correction. p values for comparisons between paediatric (onset < 15 years) and older-onset cases were corrected for the number of categories ($n = 5$). Where appropriate, 95 % confidence limits (CL) are given.

Results

Insulin-dependent diabetes mellitus (IDDM). ICA were detected in 88 of 100 cases at onset of IDDM

Table 1 Islet antibody prevalence

Patient group	Age (years)	n	ICA	GADA	Anti-37K	Anti-CPH	Anti-ICA69
IDDM							
	≤ 15	69	64 (93 %)	45 (65 %)	43 (62 %)	10 (13 %)	2 (3 %)
	> 15	31	24 (77 %)	25 (81 %)	11 (35%)[a]	3 (10 %)	0 (0 %)
Total		100	88	70	54	13	2
Pre-IDDM							
	≤ 15	10	9 (90 %)	8 (80 %)	5 (50 %)	1 (10 %)	1 (10 %)
	> 15	17	13 (76 %)	16 (94 %)	8 (46 %)	0	2 (12 %)
Total		27	22 (81 %)	24 (89 %)	13 (48 %)	1 (4 %)	3 (11 %)
Control subjects		83	1 (1 %)	1 (1 %)	0 (0 %)	1 (1 %)	1 (1 %)

[a] $p < 0.05$ vs ≤ 15 years

Table 2. Islet antibody combination

Patient group	Age (years)	n	ICA/GADA/Anti-37K				
			+/+/+	+/+/–	+/–/+	–/+/–	–/–/–
IDDM							
	≤ 15	69	23 (33 %)	21 (30 %)	20 (29 %)	1 (1 %)	4 (6 %)
	> 15	31	11 (35 %)	13 (40 %)	0 (0%)[a]	1 (3 %)	6 (19 %)
Total		100	34	34	20	2	10
Pre-IDDM							
	≤ 15	10	3 (30 %)	4 (40 %)	2 (20 %)	1 (10 %)	0
	> 15	17	7 (41 %)	5 (29 %)	1 (6 %)	4 (24 %)	0
Total		27	10 (37 %)	9 (33 %)	3 (11 %)	5 (19 %)	0
Control subjects		83	0 (0 %)	1 (1 %)	0 (0 %)	0 (0 %)	82 (99 %)

[a] $p < 0.02$ vs ≤ 15 years

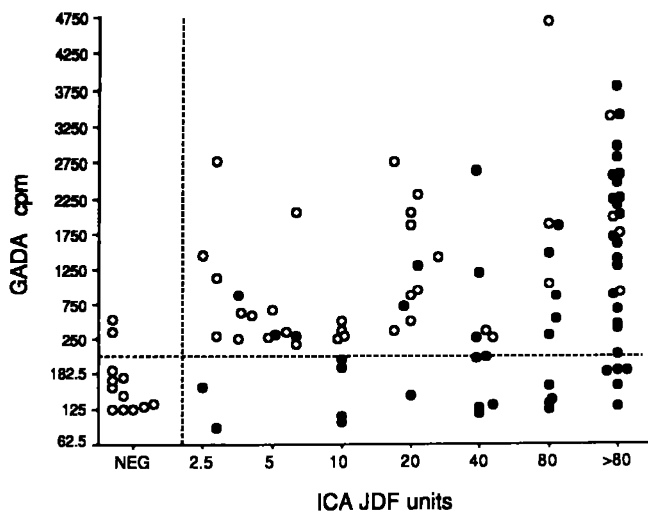

Fig. 3. Islet autoantibodies in sera from 100 newly-diagnosed IDDM patients. The filled symbols represent those in which anti-37 K were detected. The upper first percentile of 83 control sera are represented by the broken lines at 2 JDF units for ICA and 217 cpm for GADA

(CL: 80–94 %); 27 of these had between 2 and 19 JDF units and 61 ≥ 20 JDF units (Fig. 2A). ICA were the most frequently detected single humoral marker ($p < 0.01$ vs GADA; $p < 10^{-6}$ vs anti-37 K) (Table 1).

GADA were found in 70 IDDM patients (CL: 60–79 %) (Table 1; Fig. 2B), including 2 of the 12 patients without detectable ICA. ICA in the absence of GADA were found in 20 patients, all of whom had an age of onset before age 15 years ($p < 0.02$ vs > 15 years) (Table 2). There was no correlation between GADA levels and ICA titres (Fig. 3).

Anti-37 K were found in 54 patients (CL: 44–64 %); all had ICA ($p < 0.001$ vs ICA negative). They were detected more frequently in patients with disease onset before age 15 years ($p < 0.05$). Anti-37 K were strongly associated with high titre ICA and were detected in 35 of 42 (81 %) of those with ICA greater than 40 JDF units ($p < 0.002$). All 20 cases with ICA in the absence of GADA had 37 K antibodies (Table 2). Anti-37 K were not associated with the presence of GADA (Fig. 3).

All sera were tested for antibodies to CPH and ICA69. None strongly immunoprecipitated in vitro translated CPH or ICA69. Thirteen had anti-CPH levels and four anti-ICA69 levels above the upper first percentile of normal control subjects (Fig. 4).

ICA and/or GADA were detected in 90 % of cases (CL: 82–95 %). The same cases could also be identified by the presence of GADA and/or anti-37 K. Four (6 %) patients with onset before age 15 years and 6 (19 %) patients with onset after age 15 years

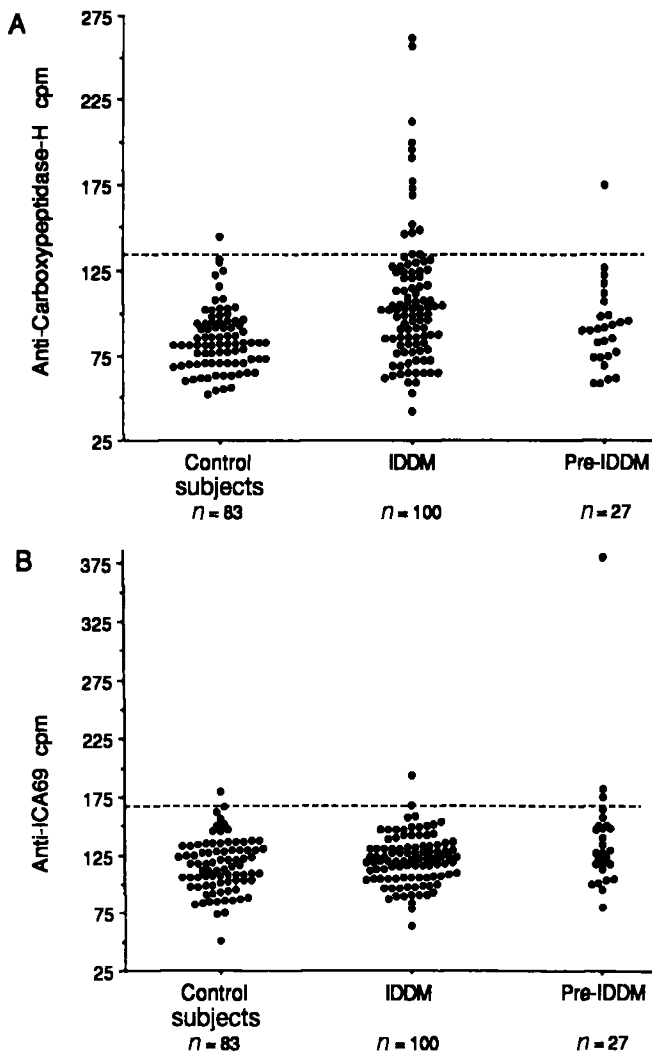

Fig. 4 (A, B). Immunoprecipitation of in vitro translated (**A**) CPH and (**B**) ICA69. The upper first percentile of control subjects is shown by the broken lines

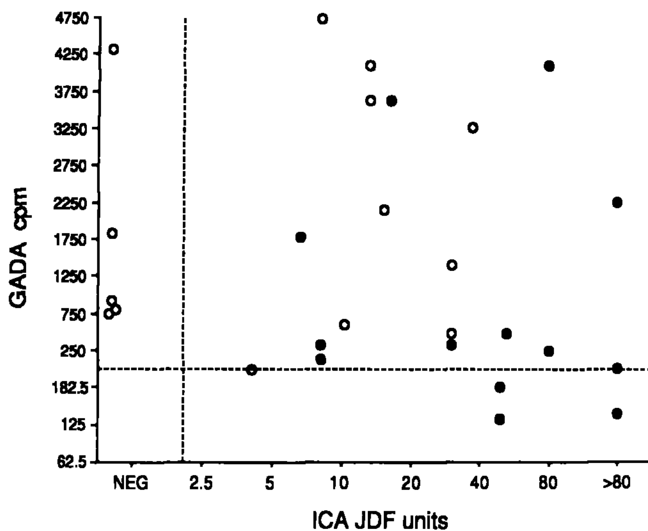

Fig. 5. Islet autoantibodies in sera from 27 pre-IDDM individuals. The filled symbols represent those in which anti-37 K were detected. The upper first percentile of 83 control sera are represented by the broken lines at 2 JDF units for ICA and 217 cpm for GADA

were negative for all three markers (Table 2). One of the antibody negative cases had IAA; none had increased levels of anti-ICA69 or anti-CPH.

Pre-IDDM. ICA and GADA were the most frequently detected single markers prior to onset of IDDM (Table 1; Fig. 5). Together they identified all 27 cases (CL: 87–100 %). Five (19 %) had GADA in the absence of ICA and three (11 %) had ICA in the absence of GADA. Anti-37 K were found in 13 cases (48 %; CL: 29–68 %), all of whom also had ICA. These included all three cases with ICA in the absence of GADA (Table 2). One (4 %) had increased levels of anti-CPH, and three (11 %) had anti-ICA69 levels above the upper first percentile of normal control subjects (Fig. 4). No significant differences between pre-IDDM and IDDM at onset were found.

Discussion

Our aim was to develop a screening method based on islet autoantibody markers that will potentially identify most future cases of IDDM. The most sensitive markers were ICA and GADA. Both were detected in 68 % of IDDM and 70 % of pre-IDDM sera, and 90 % of all IDDM and pre-IDDM sera had at least one of these markers. The majority of IDDM cases in which neither ICA nor GADA were detected were adult so that 94 % of cases with onset before age 15 had at least one of these markers as against 81 % over that age. Despite the high sensitivity of a combination of ICA and GADA, a significant proportion of patients had only one of these markers. In particular, 28 % of those with childhood-onset disease had ICA without GADA.

Anti-37 K were the next most prevalent marker in newly-diagnosed IDDM. They were found only in conjunction with ICA, and strikingly, in all cases with ICA in the absence of GADA. Here again, 90 % of IDDM cases, including 95 % of those with onset before age 15, and all pre-IDDM sera had either GADA or anti-37 K. ICA69 and CPH antibodies measured by immunoprecipitation were not detected in any of the GADA/anti-37 K negative cases, and did not increase sensitivity. IAA identified only one of these cases.

These findings suggest that ICA are the most sensitive screening test, particularly in childhood-onset diabetes, provided an assay is used that can detect low levels of antibody reproducibly. Even so, the assay is difficult to standardize, and many laboratories are unable to measure low levels consistently [7, 9]. ICA contain more than one specificity [26–29], not all of which appear to be associated with IDDM [27]. Furthermore, ICA in the absence of other islet antibody specificities are only weakly predictive of IDDM in first-degree relatives [13] or schoolchildren

[14]. In both groups increased risk is concentrated in the minority in whom more than one islet autoantibody specificity is present. In the present study, ICA were always found in association with either GADA or anti-37 K in sera taken both at and prior to diagnosis. It may therefore be preferable to undertake initial screening with those antibody specificities associated with ICA that are more closely related to disease.

GADA measurement has been proposed as a test to assess IDDM risk [12]. The assay output is numerical, removing some observer bias, and since many samples with low ICA levels have GADA levels well above the threshold for positivity used in this study, their use would avoid the difficulty of measuring low titre ICA. GADA appear to be as sensitive as ICA in adult-onset IDDM, but we found that 28 % of patients diagnosed before age 15 years have ICA in the absence of GADA at or before disease onset. This group is of particular importance in that every case had anti-37 K, a marker associated with rapid progression to insulin-requiring diabetes [13, 14, 30], thus implying an aggressive disease process. GADA measurement alone is therefore unlikely to provide as sensitive a screening test as ICA.

Our findings suggest that measuring GADA and anti-37 K could provide a test that would retain the sensitivity of ICA. Combining anti-37 K and GADA significantly enhances the sensitivity of GADA alone, and the combination of anti-37 K and ICA identifies a subgroup with markedly increased risk [13, 14, 30]. Current methods for anti-37 K are laborious and costly, and preclude the general application of this marker. Anti-37 K are however, like GADA, detected by immunoprecipitation of liquid phase antigen using small serum volumes. Therefore, provided the 37k antigen is cloned, it is likely that assays can be developed which will measure antibodies to in vitro translated recombinant GAD and 37 K antigen simultaneously. Additions of ICA69 or CPH antibodies contributed little to the analysis. Antibodies to ICA69 which have been detected in solid-phase assays [20, 24] rarely immunoprecipitate in vitro translated antigen [24], and increased binding to CPH was detected in only few sera and was not confined to GADA negative cases. IAA were not tested in all cases used in the present analysis, but we have previously found them to be absent prior to disease onset in a number of those with anti-37 K in the absence of GADA [13, 14]. Furthermore, the large serum volumes currently needed for the detection of IAA render these unsuitable for adaptation to screening assays for use in the general population.

In conclusion, we propose that a combination of GADA and anti-37 K testing could eventually replace ICA in screening for risk of IDDM. Until assays with the capacity to measure anti-37 K in large numbers of samples are available, we suggest that maximum sensitivity will be achieved by a combination of GADA and ICA. The screening strategies we propose have the potential to identify more than 90 % of future cases. We have tested this prospectively only in first degree relatives of children with IDDM in the UK, but the combinations of antibodies seen in these individuals are not different to those in the Italian non-familial cases at diagnosis, or to the few cases identified in a prospectively followed UK population of school children [14]. It is important to emphasise that the aim of the present analysis is to identify an approach to screening that maximizes sensitivity, thus identifying the greatest possible number of individuals at risk of developing IDDM within the general population. Additional or sequential tests, for example genetic analysis or metabolic testing, may then need to be applied to those with antibody markers in order to improve the specificity of prediction [31]. This approach promises in time to identify levels of risk sufficient to justify intervention trials in those with no family history of IDDM.

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