Evaluation of ICA512As in Combination With Other Islet Cell Autoantibodies at the Onset of IDDM

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OBJECTIVE — The ICA512 pancreatic islet autoantigen is a putative tyrosine phosphatase that is co-identified with the earlier described 40-kDa autoantigen. We report the frequency of autoantibodies to islet cell antigen 512 (ICA512As) in recent-onset IDDM and compare this with other islet cell autoantibodies, including those to GAD (GADAs), insulin (IAAs), and islet cell cytoplasm (ICAs) identified by immunofluorescence.

RESEARCH DESIGN AND METHODS — Sera from 232 children aged between 9 months and 14.9 years collected within 14 days of diagnosis were tested for ICA512As by a radioimmunoprecipitation assay. The results were compared with previously reported data for GADAs (n = 232), IAAs (n = 167), and ICAs (n = 230).

RESULTS — The frequency of a positive result for ICA512As in children with newly diagnosed IDDM was 60%. The frequency was greater for children with an age of onset between 5 and 10 years (69%) than for children aged <5 years (49%) and aged between 10 and 15 years (56%). The frequencies for other autoantibody reactivities were 69% for GADAs, 65% for IAAs, and 70% for ICAs. A combination of positive results for ICA512As, GADAs, and IAAs gave a sensitivity for the diagnosis of childhood IDDM of 95%, which was not significantly increased by a positive result for ICAs (96%).

CONCLUSIONS — Our results further establish that positivity in a combination of tests is more valuable for the prediction of IDDM than a result for any single autoantibody and that the age of the patient should be considered when selecting the combination of tests to use.

Since the discovery of islet cell cytoplasmic autoantibodies (ICAs) in 1974 (1), other autoantibodies to islet β-cell components have been identified as markers of underlying autoimmune insulitis. These include autoantibodies to the putative tyrosine phosphatase ICA512 (ICA512As) otherwise known as IA-2 or the 40-kDa antigen (2–4), GAD (GADAs) (5), and insulin (IAAs) (6). ICA512As, GADAs, and possibly other components contribute to ICA reactivity (7–10). Each of these autoantibody reactivities can be detected before the onset of overt IDDM (11,12).

We report here the frequency of ICA512As in sera from children with newly diagnosed IDDM and correlate these with previously ascertained frequencies for GADAs, IAAs, and ICAs. To our knowledge, this is the first report on the comparative frequencies of the four major relevant autoantibodies in recent-onset childhood IDDM.

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; GADA, GAD autoantibody; IAA, insulin autoantibody; ICA, islet cell autoantibody; ICA512A, ICA512 autoantibody; JDF U, Juvenile Diabetes Foundation unit; RU, reference unit.

RESEARCH DESIGN AND METHODS

Sera

Sera were available from 232 children with newly diagnosed IDDM ascertained through a population-based register in New South Wales, Australia, of consecutive incident cases of IDDM, as previously described (13). The mean age (± SD) at diagnosis was 8.9 ± 3.8 years (range, 0.9–14.9 years). Sera were collected within 14 days of diagnosis for the measurement of ICA512As, GADAs, IAAs, and ICAs. As test controls, sera were available from 100 healthy blood donors (mean age [range], 40 ± 14.6 [19–64] years) and from 109 nondiabetic children with illnesses other than IDDM (mean age, 10.2 ± 4.7 [0.5–19.1] years). The nondiabetic children were selected from those attending a pediatric endocrine clinic. Those with an autoimmune disease or with a family history of diabetes were excluded. A third of the subjects had a diagnosis of normal variant short stature. The remainder were diagnosed with conditions including congenital adrenal hyperplasia, postirradiation growth hormone deficiency, and dysmorphic syndromes (13).

Radioimmunoprecipitation assay for ICA512

The cDNA encoding full-length ICA512 (amino acids 1–979) was cloned into the *Eco* RI site of the Bluescript KS vector. The ICA512 protein was expressed by in vitro transcription using T3 polymerase, followed by translation in rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of 35S express label mix (Dupont, Boston, MA). The 35S-labeled ICA512 was separated from unincorporated label on a G25 Sephadex size exclusion column (Pharmacia, Uppsala, Sweden) before use in the radioimmunoprecipitation assay.

Labeled ICA512 (40,000 dpm) was incubated with 2 μl of sera diluted 1:25 in 20 mmol/l Tris, 150 mmol/l NaCl, 0.5% Triton X-100 (pH 7.4; Tris wash buffer) for 16 h at 4°C. Fifty microliters of a 50% suspension of protein A-Sepharose (Pharmacia) in Tris wash buffer was added and incubated

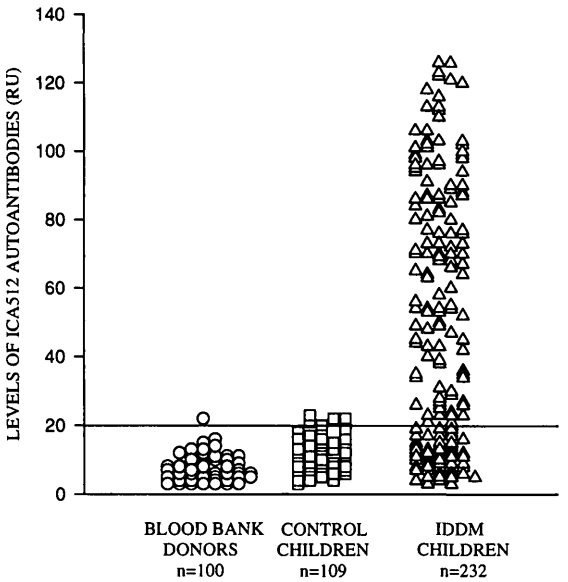

Figure 1—*Levels of ICA512As in 232 children with newly diagnosed IDDM, 100 blood donors, and 109 healthy control children. Levels of ICA512As are expressed as reference units (RUs). The dotted line at 20 RU represents the cutoff level for positivity.*

for 1 h at 4°C. The protein A-Sepharose pellet was washed four times with Tris wash buffer, resuspended in scintillant, and counted for levels of radioactivity. Autoantibody levels were quantitated by including in each assay a reference serum previously shown to have ICA512As by enzyme-linked immunosorbent assay (ELISA) (8) and defined as having 100 reference units (RUs). Autoantibody levels were expressed as a percentage of the counts immunoprecipitated by the reference serum. The levels of ICA512As in the 100 blood donors ranged from 1 to 23 RU with a mean of 6 ± 3.4 RU (mean + 3 SD = 17 RU) and levels in the nondiabetic children ranged from 1 to 23 RU with a mean of 10 ± 4.4 RU (mean + 3 SD = 24 RU). The cutoff level for positivity was established at 20 RU by taking the mean + 3 SD of the combined control groups. In the first IA-2 Proficiency Test Program (University of Florida College of Medicine, unpublished observations), the assay achieved ratings of 100% for consistency and specificity, 92% for validity, and 86% for sensitivity.

Measurement of other autoantibodies

GADAs were measured by a radioimmunoprecipitation assay using ^{125}I-labeled porcine brain GADAs as antigen (14), with levels greater than the 98th percentile (>18 RU) considered positive. In the IA-2 Proficiency Test Program (University of Florida College of Medicine, unpublished observations), the assay achieved ratings of 100% for sensitivity, specificity, consistency, and validity.

IAAs were measured on 167 of the sera by a competitive fluid-phase radioassay (13), with a cutoff level for positivity established at 33 mU/l. Sera with levels >3 SD above the mean (≥33 mU/l) were considered positive. In the IA-2 Proficiency Test Program (University of Florida College of Medicine, unpublished observations), the assay achieved ratings of 100% for sensitivity, specificity, consistency, and validity.

ICAs were measured on 230 of the sera by indirect immunofluorescence (13), with results expressed quantitatively in Juvenile Diabetes Foundation units (JDF U). Sera with ≥20 JDF U were considered positive.

Statistical analysis

Data were analyzed by the χ^2 test with Yate's correction or by Pearson's multiway χ^2 test, using Statistica for Windows, version 4.5 (StatSoft, Tulsa, OK).

RESULTS

ICA512A radioimmunoprecipitation assay characteristics

The mean (range) disintegrations per minute precipitated by the positive reference serum was 2,633 (1,630–3,590) dpm for 11 separate assays and that by six known negative sera was 188 (109–350) dpm tested repeatedly in six separate assays. The interassay coefficient of variation was 5% (n = 11) for a strongly positive control serum and varied between 23 and 36% (n = 6) for negative control sera.

Levels and frequency of ICA512As in recent-onset IDDM and control subjects

Levels of ICA512As in children with IDDM ranged from 1 to 130 RU (Fig. 1). A positive result was obtained in 140 of the 232 (60%) sera tested (Table 1). The frequency was greatest among children with an age of onset between 5 and <10 years (69%), compared with those aged <5 years (49%) and those aged 10–15 years (56%) (Table 1). A weakly positive result (20–23 RU) was given by five (4.5%) sera from nondiabetic children and one of the 100 (1%) sera from blood donors.

Frequency of other autoantibodies and the effect of age

Frequencies of GADA, IAA, and ICA reactivities are from previously published data (13). A positive result for GADAs was present in 161 of the 232 (69%) sera tested, a positive result for IAAs was present in 108 of the 167 (65%) sera tested, and a positive result for ICAs was present in 160 of the 230 (70%) sera tested (Table 1).

The frequency of GADA positivity was more prevalent among children with an age of onset between 10 and 15 years (74%) than among children aged <5 years (66%) or between 5 and <10 years (65%). The frequency of IAA positivity was most prevalent among children with an age of onset <5 years (90%), and there was a significant decline in frequency with increasing age to 71% in cases aged between 5 and <10 years and, further, to 50% in those aged between 10 and 15 years (P = 0.0003; Pearson's χ^2, df = 2). The frequency of ICAs was greatest among children with an age of onset between 5 and <10 years (77%), compared with children aged <5 years (71%) or between 10 and 15 years (63%) (Table 1). Of the 232 sera tested, 18 (8%) were autoantibody negative (data not shown).

Frequency of autoantibody combinations and autoantibody number

Sera that were tested by all four assays were used to evaluate autoantibody number and the sensitivity of combinations of tests. The majority of children were positive for more than one autoantibody (Fig. 2). Of the 166

Table 1—*Summary of the data for children with newly diagnosed IDDM and control sera*

Group	*n*	M/F	Mean age (years)	Age range (years)	ICA512A+	IAA+	GADA+	ICA+
IDDM patients (age of onset)								
<5 years	41	18/23	2.7 ± 1	0.9–4.6	22/41 (49)	26/29 (90)*	27/41 (66)	29/41 (71)
5–10 years	83	43/40	7.6 ± 1.4	5.0–9.9	57/83 (69)	47/66 (71)	54/83 (65)	64/83 (77)
10–15 years	108	54/54	12.4 ± 1.2	10–14.9	61/108 (56)	36/72 (50)	80/108 (74)	67/106 (63)
Total	232	115/117	8.9 ± 3.8	0.9–14.9	140/232 (60)	109/167 (65)	161/232 (69)	160/230 (70)
Control subjects								
Children	109	50/59	10.2 ± 4.7	0.5–19.1	5/109 (4.5)	0/90 (0)	3/109 (3)	0/39 (0)
Blood donors	100	NA	40 ± 14.6	19–64	1/100 (1)	NA	NA	NA

Data are *n* (%) or means ± SD. *The decrease in the frequency of IAAs with the increase of age was significant ($P = 0.0003$). NA, not available.

sera, 19 (11%) were seropositive for 1 autoantibody, 31 (19%) for two autoantibodies, 68 (41%) for three autoantibodies, and 41 (25%) for four autoantibodies (Fig. 2). Thus, 85% (140/166) were seropositive for two or more autoantibodies.

The sensitivity for the diagnosis of IDDM in this set of children was markedly increased when the test for GADAs was used in addition to the tests for other IDDM-related autoantibodies (Table 2). Thus, the test for GADAs in addition to IAAs increased positivity to 97% in children with an age of onset <5 years and to 90% in children with an age of onset between 10 and 15 years. The test for ICA512As in addition to GADAs and IAAs increased positivity to 100% in children with an age of onset <5 years and to 93% in children with an age of onset between 10 and 15 years (Table 2). Tests for GADAs in addition to testing for ICA512As increased positivity to 91% in children with an age of onset between 5 and <10 years and 94% in children with the addition of a positive test result for IAAs (Table 2). The inclusion of a positive result for ICAs with tests for the other three autoantibodies did not significantly increase positivity among children for any age group (Table 2). Of the children positive for ICAs, seven (5%) were negative for ICA512As and GADAs, and five of these were positive for IAAs (data not shown).

CONCLUSIONS — We describe the frequency of ICA512As, relative to GADAs, IAAs, and ICAs, in Australian children with recent-onset IDDM and in nondiabetic subjects. The sensitivity for ICA512As was 60%, and the specificity was 99%, according to tests on 100 blood donors, and 96%, according to tests on 109 nondiabetic children.

Assays reported for ICA512As vary in frequency of positivity. The initial assay was an ELISA in which the antigen used was the intracellular protein tyrosine phosphatase-like domain expressed in *Escherichia coli* (15). Approximately 50% of children with newly diagnosed IDDM were seropositive by this assay (2,8). Subsequently, several radioimmunoprecipitation assays have been described with frequencies of positivity in recent-onset IDDM of 66% of 50 subjects (9), 53% of 100 subjects (3), and 38% of 42 subjects (16) and in preclinical diabetes of 48% of 33 subjects (16). These differences could be due to differences in assay procedure (ELISA versus radioimmunoprecipitation) or in the cDNA constructs used to express the ICA512 protein. The study by Lan et al. (9) used the full-length protein (amino acids 1–979), whereas that of Gianani et al. (16) used a 548–amino acid fragment encoding the intracellular domain, and the original ELISA used the putative intracellular domain (15). Differences in the frequency of positivity in the reported assays could also depend on the age of onset and the duration of IDDM, as shown in our previous study (8).

Our analysis of the results of the tests using multiple autoantigens in children with recent-onset IDDM further establishes that testing for the presence of autoantibodies to more than one autoantigen should increase the sensitivity for diagnosis (13,17–21). We recognize that data at disease onset are not necessarily applicable to prediction, but we note that GADAs, ICA512As, and even ICAs are not transient reactivities and persist for some months or, in the case of GADAs, for years after the onset of IDDM (14,22,23). Thus, it is likely that the frequencies of these autoantibodies at the onset of IDDM would be representative of the frequencies immediately before onset. In prediabetic subjects, a positive result for two or more autoantibodies has proven to be more predictive of IDDM (17,24,25). In particular, Verge et al. (24) tested 882 first-degree relatives for ICA512As, GADAs, and IAAs; of

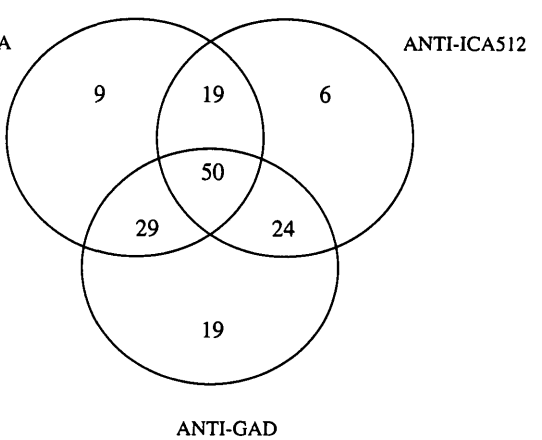

Figure 2—*Frequency of positivity to IAAs, GADAs, and ICA512As in children with recent-onset IDDM. Sera not tested for all four autoantibodies were excluded from the analysis* (n = 166).

Table 2—*Frequency of positivity using various combinations of autoantibodies in children with recent-onset IDDM*

Autoantibody or autoantibody combination	Age at onset			
	<5 years	Between 5 and 10 years	10–15 years	Total
ICA	22/29 (76)	52/66 (78)	44/72 (61)	118/167 (71)
ICA512	16/29 (55)	44/66 (67)	41/72 (57)	101/167 (60)
GAD	18/29 (62)	46/66 (70)	58/72 (80)	122/167 (73)
IAA	26/29 (90)	47/66 (71)	36/72 (78)	143/167 (65)
ICA512 or IAA	26/29 (90)	61/66 (91)	56/72 (78)	143/167 (86)
ICA512 or GAD	21/29 (72)	57/66 (86)	68/72 (94)	146/167 (87)
GAD or IAA	27/29 (93)	62/66 (94)	62/72 (86)	151/167 (90)
ICA512 or IAA or GAD	29/29 (100)	62/66 (94)	69/72 (96)	160/167 (95)
ICA or ICA512 or IAA or GAD	29/29 (100)	62/66 (94)	70/72 (97)	161/167 (96)

Data are *n* (%). Sera not tested for all four antibodies were excluded from the analysis.

those who developed diabetes, 98% were seropositive for one or more of these autoantibodies, and for relatives seropositive for at least two autoantibodies, the risk of diabetes within 3 years was 39% and within 5 years 68%. Our current study shows that 96% of children with IDDM were seropositive for at least one of ICA512As, GADAs, IAAs, or ICAs, with 85% seropositive for two or more autoantibodies, and that optimal combinations of tests could be recommended according to age of the subjects tested. Thus, GADAs, combined with IAAs, were more sensitive indicators in children with an age of onset <5 years, whereas GADAs, combined with ICA512As and IAAs, were more sensitive in children with an age of onset >5 years. If population screening were to include children <5 years of age, tests for IAAs should be included, since IAAs detected 90% of children with IDDM in this age group.

A positive result for ICA512As and GADAs in combination gave a much higher sensitivity (88%) than did the ICA test alone (72%). In our study, the inclusion of a positive result for ICAs did not significantly increase the sensitivity when combined with ICA512As and GADAs. The main reason for this was that a positive ICA test is mostly dependent on the presence of autoantibodies against ICA512 or GAD, as shown previously in other studies (7–9,26). The relatively high cutoff level used (20 JDF U) would have resulted in some loss of sensitivity. There were 11 sera with levels ranging between 10 and 20 JDF U, so that if the cutoff level had been lowered to 10 JDF U, the sensitivity would have been 77%, but still lower than the combination of ICA512As and GADAs. In this study, 5% of the children were seropositive for ICAs, yet gave a negative result for ICA512As and GADAs, and 3% of these children were seropositive for IAAs. A recent study by Dotta et al. (10) reported that 64.5% of ICA-positive relatives of subjects with IDDM gave a positive result for antipancreatic GM2-1 ganglioside, suggesting that autoantibodies to other islet cell components may also contribute to ICAs. However, since assays now available to test for GADAs and ICA512As are reliable and convenient to use and, unlike the ICA test, readily quantifiable, the use of a combination of ICA512A and GAD tests can be recommended in preference to ICAs.

In conclusion, the frequency of ICA512As detected in children with recent-onset IDDM in this study was 60%. The analysis of all four autoantibodies revealed that each detects an overlapping group of individuals and that the frequencies of the combinations of autoantibodies may be dependent on the age of subjects tested. Therefore, combination of autoantibody tests can be recommended in preference to testing for one autoantibody alone for screening purposes.

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