Two-Step Islet Autoantibody Screening for Risk Assessment of Type 1 Diabetes in Relatives

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OBJECTIVE — To examine the performance of islet cell antibodies (ICAs) and antibodies to glutamate decarboxylase (GADA), IA-2 (IA-2 antibody [IA-2A]), and insulin (insulin autoantibody [IAA]), alone and in combination, in assessing type 1 diabetes risk within type 1 diabetic families to identify a practical and effective screening strategy for predicting type 1 diabetes in relatives.

RESEARCH DESIGN AND METHODS — ICA, GADA, IA-2A, and IAA were determined in 806 first-degree relatives participating in a prospective type 1 diabetes family study (median follow-up 6.17 years, range 0.6–8.3). The conferred risk of developing type 1 diabetes within 6 years was evaluated by Kaplan-Meier for each antibody marker, used alone or in combination.

RESULTS — ICAs were detected in 3%, GADA in 5.1%, IA-2A in 2.5%, and IAA in 3.7% of relatives; ≥1 antibody markers were detected in 10.7% of relatives and ≥2 were detected in 1.9% of relatives. The risk of type 1 diabetes at 6 years was 1.5% in relatives with only 1 marker and 24.8% in relatives with ≥2 markers. As a practical and effective strategy for type 1 diabetes risk assessment in relatives, this study indicates a first-step screening based on GADA and IA-2A measurement—which identified 6.5% of relatives, including all who developed the disease, with a 6-year type 1 diabetes risk of 9.0%—followed by a second step based on ICA and IAA measurement in relatives with either GADA or IA-2A, which identified a total of 1.9% of all relatives as having ≥2 markers, and a 6-year risk of 24.8%, including 6 of 7 who developed type 1 diabetes.

CONCLUSIONS — A two-step antibody screening, based first on GADA and IA-2A and then on ICA and IAA measurements in identified individuals, is likely to be a practical, sensitive, and effective strategy for predicting type 1 diabetes in first-degree relatives.

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The clinical onset of type 1 diabetes is preceded by the appearance in the circulation of islet autoantibodies (1). These may be detected by indirect immunofluorescence on human pancreatic cryosections as islet cell antibodies (ICAs), the classical serological marker of the disease (2). ICA is the most sensitive single antibody marker of type 1 diabetes (3,4), is the best validated in prediction studies (5,6), and represents the primary test for recruitment into intervention trials (7,8). However, ICA measurement is technically laborious, difficult to standardize (9), and therefore not ideal for large-scale screening.

Other islet-specific humoral markers are now antigenically defined and include autoantibodies to insulin (IAA) (10), antibodies to glutamate decarboxylase (GADA) (11), and the protein tyrosine phosphatase-like IA-2 (IA-2A) and IA-2β molecules (12,13). GADA and IA-2A are also components of the ICA detected by indirect immunofluorescence (14–17). Molecular cloning of target antigens has allowed the development of simple, sensitive, high-throughput immunoassays for antibody measurement (16,18). A combined use of these three antibody markers offers the potential for replacing ICA measurement in the identification and prediction of type 1 diabetes.

In this study we examined the performance of ICA, GADA, IA-2A, and IAA, alone and in combination, in assessing type 1 diabetes risk within type 1 diabetic families to identify a practical and effective screening strategy for predicting type 1 diabetes in relatives. With a two-step strategy using all four antibody markers, accurate prediction of type 1 diabetes appears possible if two or more antibody markers are detected after primary screening with GADA and IA-2A measurement and ICA and IAA subsequently measured in those with either GADA or IA-2A.

RESEARCH DESIGN AND METHODS

Subjects

First-degree relatives of type 1 diabetic patients. Between February 1989 and October 1995, 268 families were enrolled in the prospective hospital-based San Raffaele Type 1 Diabetes Family Study. All families resided within the area of Milan, Northern Italy, and had at least one proband who developed type 1 diabetes before the age of 30 and at least one unaffected sibling under the same age. Informed consent was obtained from all individuals before entering the study. Within 3 months from the onset of type 1 diabetes in the proband, 95 families were sampled;

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Abbreviations: GADA, glutamate decarboxylase antibody; IA-2A, IA-2 antibody; IAA, insulin autoantibody; ICA, islet cell antibody; JDF, Juvenile Diabetes Foundation.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Prevalence and number of antibody markers*

	n	ICA	GADA	IA-2A	IAA	≥1 antibody	1 antibody	≥2 antibodies
Relatives	806	24 (3.0)	41 (5.1)	20 (2.5)	31 (3.7)	86 (10.7)	70 (8.7)	16 (2.0)
Parents	470	9 (1.9)	15 (3.2)	6 (1.3)	12 (2.6)	38 (8.1)	35 (7.4)	3 (0.6)
Siblings	336	15 (4.4)	26 (7.7)*	14 (4.2)	19 (5.4)	48 (14.3)	35 (10.4)	13 (3.9)*
Probands	97	76 (78.3)	70 (72.2)	51 (52.6)	36 (37.1)	87 (89.7)	11 (11.3)	76 (78.3)

Data are *n* or *n* (%). *$P < 0.05$ vs. parents.

the remaining 173 families were sampled at a median of 55 months (range 4–312) after type 1 diabetes onset in the proband. A total of 806 nondiabetic first-degree relatives were investigated: 423 were female and 383 were male; 470 were parents (246 mothers, 224 fathers; mean age 46.6 ± 8.8 years, range 24–78) and 336 were siblings (177 females, 159 males; mean age 18.9 ± 9.5 years, range 2–48). A blood sample was obtained from all participants at the time of entry, and serum was stored at −20°C until antibody measurement. ICA, GADA, IA-2A, and IAA were measured in all first-degree relatives. All relatives were observed from the initial sampling to the end of June 1997, unless type 1 diabetes was diagnosed before that date. At the time of analysis, the mean follow-up was 5.42 ± 2.12 years (median 6.17, range 0.6–8.3 years). Serum samples obtained within 2 weeks from the first insulin injection were also available from 97 type 1 diabetic probands from the families studied. Sixty were male patients, and the mean age was 15.9 ± 9.0 years (range 1–30). Serum samples from the remaining probands, having been obtained later in the course of disease, were not suitable for IAA measurement and therefore were not considered in this study. Type 1 diabetes was defined according to the World Health Organization criteria, and all patients were placed on insulin therapy at the time of diagnosis and remained insulin-dependent thereafter.

Autoantibody measurements

ICA. These antibodies were measured in undiluted sera by indirect immunofluorescence on a 4-μm cryostat section of blood group O human pancreas, as previously described (2). Samples with ICA were subsequently quantified in Juvenile Diabetes Foundation (JDF) units by end-point titration using doubling dilutions in 10 mmol/l phosphate-buffered saline in parallel with a local standard calibrated to 2.5, 5, 10, 20, 40, and 80 JDF units. Endpoint titers of test samples were converted to JDF units by comparison with a standard curve of $\log_2$ JDF units versus $\log_2$ of endpoint titer of the standard sera (5). The threshold of ICA detection ranged throughout the study between 2.5 and 5 JDF units as determined by the lowest standard detectable. This ICA assay had a sensitivity of 86%, a specificity of 98%, and reproducibility of 100% at the last Combined Islet Autoantibody Workshop (19).

GADA and IA-2A. Measurements were performed by radiobinding assay with in vitro translated ^{35}S-methionine labeled GAD65 or IA-2, as previously described (5,16,18). Results were converted into arbitrary units by extrapolation from a standard curve with a local standard, designated 100 units, undiluted and at 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 in negative serum. The thresholds for positivity were determined from the 99th percentile of 178 control subjects (median age 12 years, range 1–40) and corresponded to 3 units for GADA and 1.5 units for IA-2A. These GADA and IA-2A assays obtained the following performances at the last Combined Islet Autoantibody Workshop: GADA, sensitivity 88%, specificity 98%, reproducibility 100%; IA-2A, sensitivity 70%, specificity 99%, reproducibility 100% (19).

IAA. These antibodies were measured using a radiobinding assay, as previously described (20). Briefly, 80 μl of acid charcoal-extracted serum samples were incubated overnight at 4°C with constant amounts of Tyr-A-14 mono-iodinated human insulin (~20,000 cpm, specific activity ~250 μCi/μg). Insulin immune complexes were precipitated with polyethylene glycol, and radioactivity was counted in a γ-counter. The results were expressed as difference between percentage of binding (Δ%) in the absence and presence of an excess of cold insulin (5.58 μmol/l). Threshold for positivity was established at the 99th percentile of 330 control subjects, corresponding to 0.95 Δ%. The IAA assay used in this study was tested in several workshops and proficiency test evaluations, with a sensitivity ranging from 64 to 100%, a specificity from 93 to 100%, and a reproducibility from 91 to 100%.

ICA competition studies

Sera from relatives and probands with elevated levels of ICA and only one of IA-2A and GADA were retested in the ICA assay after competition with recombinant IA-2 or GAD65. An octyl-glucoside lysate of *E. coli* transformed with pTRC His (Invitrogen, San Diego, CA) IA2–2 or pTCR His ICA_{69} plasmid (control) was prepared and dialyzed as previously described (16). Purified recombinant GAD65 was prepared as previously described (21). Sera (10 μl) were incubated with IA-2 preparation (10 μl), purified GAD65 (10 μl), or control ICA_{69} lysate (10 μl) for 1 h at 4°C and then tested in the ICA assay. A polyclonal mouse anti–IA-2 antiserum and a serum of a patient with stiff-man syndrome and high titer of GAD65 antibodies were included as controls. Subjects in whom ICA reactivity was completely abolished by competition with GAD65 or IA-2 were considered to have only one antibody in survival analyses of number of antibodies.

Statistical analysis

Differences in antibody frequency between groups were evaluated using the χ^2 test with Yate's correction or the Fisher's exact test, when appropriate. Differences in antibody titers between groups were evaluated using the Mann-Whitney *U* test. Sensitivity of antibody markers, alone or in combination, was calculated as percent of type 1 diabetic probands above the established cutoff. The survival rates, projected risks, and 95% CIs were calculated by Kaplan-Meier analysis, and comparison between groups was performed using the log-rank test.

RESULTS

Antibody prevalence

The prevalences of elevated antibody levels for ICA, GADA, IA-2A, and IAA in first-degree relatives and type 1 diabetic pro-

Table 2—*Association of antibody markers and progression to type 1 diabetes in first-degree relatives*

ICA	GADA	IA-2A	IAA	*n*	Diabetes	
+	+	+	+	4	2	≥2 antibodies
+	+	+	−	4	2	
+	+	−	+	2	1	
+	+	−	−	2*	1	
+	−	+	−	1	0	
−	+	−	+	2	0	
−	+	+	−	1	0	
+	−	−	−	11	0	1 antibody
−	+	−	−	26	1	
−	−	+	−	10	0	
−	−	−	+	23	0	
−	−	−	−	720	0	0 antibodies

The ICA column includes all ICAs. *Includes one subject with ICAs that were completely inhibited by competition with GAD65. Progression to type 1 diabetes was observed in the subject in whom ICA could not be inhibited by GAD65.

bands are reported in Table 1. Prevalences in relatives ranged between 2.5% (IA-2A) and 5.1% (GADA), and 10.7% had at least one antibody marker. Higher prevalences were found in siblings than in parents, reaching statistical significance in the case of GADA ($P < 0.05$). In probands, antibody prevalences ranged between 37.1% (IAA) and 78.3% (ICA); 89.7% had at least one antibody marker. IAAs were significantly more frequent in probands aged ≤14 years than in those aged 15–30 years (59.0 vs. 24.1%, respectively; $P < 0.02$).

Number and levels of antibodies

Of relatives identified as having islet antibodies, only a minority had more than one antibody marker: 70 (8.7%) had one, 6 (0.7%) had two, 6 (0.7%) had three, and 4 (0.5%) had all four antibody markers. The majority of relatives with elevated levels of only one antibody marker had either GADA or IAA (Table 2). In contrast, 11 (11.3%) probands had one marker only, whereas 26 (26.8%) had two, 32 (33%) had three, and 18 (18.6%) had all four antibody markers (Table 1).

Sera from 2 relatives and 14 probands had elevated levels of ICA and GADA only; and 1 relative and 6 probands had elevated ICA and IA-2A only. Competition with recombinant GAD65 abolished ICA in 1 of 2 relatives and in 5 of 14 probands with ICA and GADA only; the recombinant IA-2 did not inhibit ICA reactivity of the single relative and completely inhibited ICA staining in 1 of 6 probands with ICA and IA-2A. Recombinant GAD65 completely inhibited ICA staining by the serum from the stiff-man syndrome patient with high-titer GAD65 antibodies, and recombinant IA-2 preparation completely inhibited ICA staining of the mouse polyclonal anti–IA-2 serum.

Levels of antibodies were significantly higher in relatives with ≥2 antibodies than in those with 1 antibody for ICA ($P < 0.01$), GADA ($P < 0.01$), and IA-2A ($P = 0.025$), whereas IAA levels were comparable in the two groups of relatives.

Of the 15 relatives having ≥2 antibodies, 12 have had follow-up samples: in 10 relatives, ≥2 antibodies were persistently detected, and in 2 relatives, only 1 antibody was detected during follow-up. Of the 71 relatives having only one antibody, 14 have been retested; antibodies were detected in follow-up samples from 7 of them.

Antibody combinations and progression to type 1 diabetes

Seven (0.9%) relatives (6 siblings and 1 parent) developed type 1 diabetes at 0.92, 1.42, 2, 3.08, 6.33, 6.58, and 7.67 years after entry into the study. All but one relative had more than one antibody marker. All those developing the disease had GADA, six had ICA, four had IA-2A, and three had IAA (Table 2). All but one have had follow-up samples and were persistently antibody positive.

The one relative in whom ICA was inhibited by GAD65 was 19 years of age, has been followed for 3.7 years, has not developed type 1 diabetes, and has a normal intravenous glucose tolerance test at 3.7 years of follow-up.

Risk of type 1 diabetes

The projected risks for relatives of developing type 1 diabetes within 6 years for individual, combined, and number of anti-

Table 3—*Type 1 diabetes risk at 6 years for individual, combined, and number of antibody markers*

Antibody marker(s)	Relatives	6-year risk	Parents	6-year risk	Siblings	6-year risk
ICA	24 (3.0)	11.6 (0–27.9)	9 (1.9)	0	15 (4.4)	26.0 (0.7–51.9)
GADA	41 (5.1)	11.5 (0.7–22.2)	15 (3.2)	0	26 (7.7)	18.2 (1.8–34.5)
IA-2A	20 (2.5)	12.6 (0–29.0)	6 (1.3)	0	14 (4.2)	17.9 (0–40.9)
IAA	31 (3.7)	3.4 (0–10.1)	12 (2.6)	0	19 (5.4)	5.9 (0–17.1)
GADA or IA-2A	52 (6.5)	9.0 (0.5–17.6)	20 (4.3)	0	32 (9.5)	14.6 (1.2–28.1)
GADA, IA-2A, or IAA	74 (9.2)	6.0 (0.3–11.7)	30 (6.4)	0	44 (13.1)	10.2 (0.5–19.9)
GADA, IA-2A, or ICA	63 (7.8)	7.3 (0.4–14.1)	27 (5.7)	0	36 (10.7)	12.9 (0.9–24.8)
GADA, IA-2A, ICA, or IAA	86 (10.7)	5.2 (0.2–10.1)	38 (8.1)	0	48 (14.3)	9.3 (0.5–18.1)
0 antibodies	720 (89.3)	0	432 (91.9)	0	288 (85.7)	0
1 antibody only*	71 (8.8)	1.5 (0–4.3)	35 (7.4)	0	36 (10.7)	2.9 (0–8.4)
≥2 antibodies	15 (1.9)	24.8 (0.2–49.5)†	3 (0.6)	0	12 (3.6)	31.4 (1.7–61.1)†

Data are *n* (%) or % (95% CI). *Those subjects with ICA and only GADA or IA-2A in whom ICA was completely inhibited by competition with GAD65 or IA-2 were considered to have only 1 antibody. †$P < 0.001$ vs. 1 antibody only.

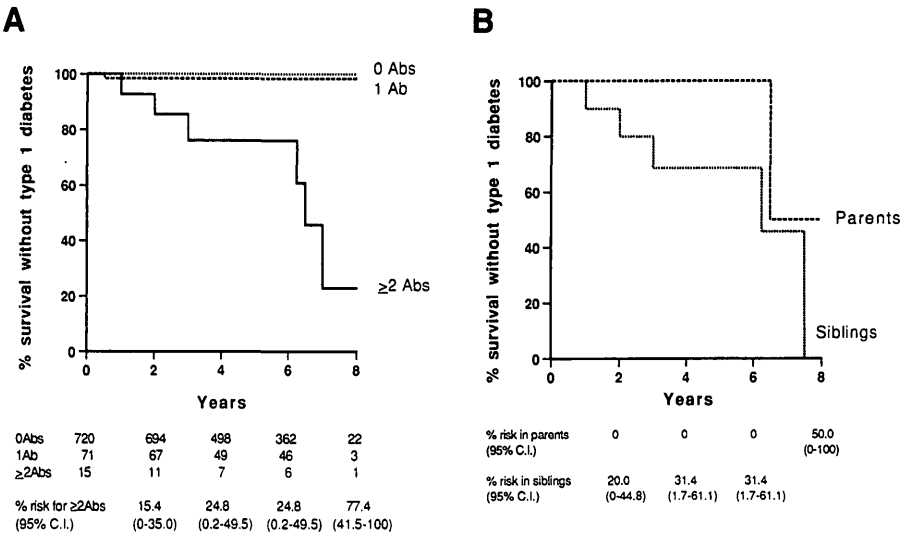

Figure 1—A: *Survival without type 1 diabetes in relatives by number of antibodies ($P < 0.01$ for ≥2 antibodies vs. 1 antibody).* B: *Survival without type 1 diabetes in parents and siblings with ≥2 antibodies (NS).*

body markers are reported in Table 3. The presence in relatives of one single elevated antibody marker did not confer a significant risk compared with those with no detectable antibodies. Relatives with two or more elevated antibodies had a significantly higher risk compared with those with one elevated antibody ($P < 0.001$) (Fig. 1).

Screening strategies

A combination of GADA and IA-2A identified 6.5% of relatives or 9.5% of siblings, including all seven who developed type 1 diabetes, as well as 83.5% of probands at disease onset (Table 3). In probands, this combination as a primary screening test was more sensitive than ICA measurement alone. The addition of IAA and/or ICA only marginally increased sensitivity in probands. The performance of a two-step screening strategy based on primary testing with GADA and IA-2A followed by additional testing for ICA and IAA in those with either GADA or IA-2A is reported in Table 4. The detection of two or more of the four antibody markers in this strategy identified 1.9% of relatives (3.6% of siblings) carrying a risk of 24.8% (31.4% in siblings) of developing type 1 diabetes within 6 years, including six of seven of those who developed the disease. Those with only one marker in this strategy, i.e., only GADA or IA-2A, had a significantly lower risk, not different from baseline (Table 4).

CONCLUSIONS — Risk assessment of type 1 diabetes has been mainly based on the measurement of ICA (5,6,22,23). However, the relative labor intensity and difficulty in standardizing measurement in the immunofluorescence assay render this antibody marker less than ideal for large-scale screening. GADA, IA-2A, and IAA are antibody markers of type 1 diabetes measurable in radiobinding assays and are candidate markers to replace ICA for screening purposes (24–27). Our study analyzes all four markers as screening tests in a cohort of first-degree relatives of type 1 diabetic patients followed up to 8 years.

The purpose of this study was to identify a strategy that may replace ICA as the primary antibody screening test. Our findings indicate that a combination of GADA and IA-2A is likely to achieve a performance in first-degree relatives that is comparable with that of ICA. The prevalence in relatives of the three antigen-specific antibody markers tested in the study ranged between 2.5 and 5.1%, with associated risks for developing type 1 diabetes within 6 years of 3.4 to 12.6%. Based on the pre–type 1 diabetes cases, which are few, GADA alone appears to be sufficient as a primary screening test, because all had elevated levels of this marker. A recent study of larger numbers of pre–type 1 diabetic relatives showed that although GADA was sensitive, it did not identify all cases (24). In agreement with this, only 72% of a much larger number of probands had GADA at disease onset. The addition of IA-2A significantly increased the sensitivity to above that of ICA alone. Screening with GADA and IA-2A identified 6.5% of all relatives studied. GADA and IA-2A represent a useful screening combination because their measurement is based on similar assays, allowing combined detection of both antibody markers in a single assay (16). The additional measurement of IAA in primary screening identified only one proband who did not have GADA or IA-2A. However, in view of the much higher sensitivity of IAA before type 1 diabetes onset in young relatives and the availability of new microassays (28), the addition of IAA may prove suitable for sensitive screening in young individuals.

Selection of those with the highest risk for type 1 diabetes is based on titration of antibody level (5,23) and the detection of multiple antibody markers (24–27). In this study, >10% of relatives had elevated anti-

Table 4—*Two-step antibody screening strategy*

Antibody marker(s)	Relatives	6-year risk	Siblings	6-year risk
Stage 1				
GADA and IA-2A				
0 antibodies	754 (93.5)	0	304 (90.5)	0
≥1 antibodies	52 (6.5)	9.0 (0.5–17.6)	32 (9.5)	14.6 (1.2–28.1)
Stage 2 in those with ≥1 antibodies				
Strategy A: IAA and ICA				
1 antibody	37 (4.6)	2.8 (0–8.2)	20 (5.9)	5.3 (0–15.3)
≥2 antibodies	15 (1.9)	24.8 (17.2–49.4)	12 (3.6)	31.4 (1.7–61.1)
Strategy B: IAA only				
1 antibody	39 (4.8)	5.3 (0–12.3)	21 (6.2)	10.0 (0–23.2)
≥2 antibodies	13 (1.6)	19.2 (0.43.2)	11 (3.3)	23.8 (0–52.8)

Data are *n* (%) or % (95% CI). For strategy A, those subjects with ICA and only GADA or IA-2A in whom ICA was completely inhibited by competition with GAD65 or IA-2 were considered to have only 1 antibody.

body levels, but the majority of these had only one antibody marker, the presence of which was not associated with a significantly increased risk for developing type 1 diabetes. In contrast, a markedly elevated risk was observed in the minority of relatives with increased levels of two or more antibody markers, who also had higher titers of ICA, GADA, and IA-2A. This is in accordance with previous reports showing that the combination of multiple autoantibody markers improves the effectiveness of type 1 diabetes risk assessment in first-degree relatives (24–27). The additional measurement of ICA and IAA in those relatives with either GADA or IA-2A also improved the ability to predict type 1 diabetes. Those with two or more antibody markers using this strategy had a markedly increased risk compared with those in whom only GADA or IA-2A were detected. The use of ICA as an additional marker may be questioned because of the contribution of both GADA and IA-2A to ICA reactivity. However, ICA in the majority of relatives and probands with ICA and only one of GADA or IA-2A could not be abolished by competition with GAD65 or IA-2. Moreover, the exclusion of ICA in the screening strategy is likely to reduce sensitivity, especially in individuals with onset above 15 years of age in whom, in contrast to younger patients, it has been shown that both IA-2A and IAA are relatively infrequent at disease onset (16,27,29). The inability to completely inhibit ICA with GAD65 and IA-2 also strongly suggests the presence of additional islet antibodies in type 1 diabetic patients. Their identification may further improve type 1 diabetes risk assessment.

An interesting observation of this study is the low rate of progression to type 1 diabetes in relatives, markedly lower than that of relatives in other regions (30,31). In accordance with this is the low prevalence (2.9%) of newly diagnosed type 1 diabetic patients with an affected first-degree relative found in the EURODIAB survey for the Lombardy region (R.B., F.M., unpublished observations). Lombardy also has one of the lowest incidence rates of type 1 diabetes in Europe (32,33), and the risk of type 1 diabetes in first-degree relatives may parallel the overall disease risk in the region (34). Despite an apparent lower overall risk, the prevalence of antibody markers in our cohort was similar to that found in other regions (5,6,26,31,35,36), indicating that the risk associated with the detection of antibody markers in first-degree relatives may differ depending on factors such as ethnicity and geographical location. These observations are consistent with Bayes' principles.

In summary, our study suggests that the use of ICA measurement in primary screening for pre–type 1 diabetes in affected families can be replaced by measurement of antigen-specific markers. The use of GADA and IA-2A appears to be a suitable replacement, because they achieve a sensitivity equal to or greater than that of ICA alone and can be measured in relatively simple radiobinding assays. Screening is likely to be rendered more efficient by the use of assays that combine measurement of both GADA and IA-2 (16,37) and by measurement of antibodies in less invasive and more economical capillary blood samples. Measurement of other antibody markers such as IAA and ICA in those with GADA or IA-2A allows the selection of relatives with the greatest risk for type 1 diabetes. Such strategies should markedly improve our ability and capacity to identify pre–type 1 diabetic first-degree relatives, which is an important prerequisite for the evaluation of potential early intervention therapies.

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