Prediction of Type I Diabetes in First-Degree Relatives Using a Combination of Insulin, GAD, and ICA512bdc/IA-2 Autoantibodies

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Islet cell antibodies (ICAs) are predictive of type I diabetes in first-degree relatives, but this immunohistochemical assay has proven difficult to standardize. As an alternative, we assessed the use of radioassays for antibodies against three molecularly characterized islet autoantigens, including ICA512bdc (amino acid residues 256–979 of the IA-2 molecule, incorporating the intracellular domain). We measured insulin autoantibodies (IAAs), GAD autoantibodies (GAAs), and ICA512bdc autoantibodies (ICA512bdcAAs) by radioassay, in addition to ICAs, in 882 first-degree relatives of patients with type I diabetes, 50 of whom later developed diabetes with a median follow-up of 2.0 years (maximum 11.3 years). The cutoff for each radioassay was determined by testing >200 control subjects. When autoantibody frequencies among the relatives were analyzed according to relationship to the proband, the offspring of diabetic fathers had a higher frequency of ICA512bdcAAs ($P = 0.008$), IAAs ($P = 0.0001$) and GAAs ($P = 0.0001$) than the offspring of diabetic mothers. ICA512bdcAAs and IAAs both showed a significant association with HLA-DR4-DQ8 ($P = 0.0005$). Among relatives developing diabetes, 98% had one or more of IAAs, GAAs, or ICA512bdcAAs, and 80% had two or more of these autoantibodies, compared with none of the control subjects. Using survival analysis to allow for different lengths of follow-up, there was a significant increase in the risk of diabetes with the number of these autoantibodies present, comparing zero, one, two, and three autoantibodies ($P < 0.0001$, log-rank test), and by Cox regression analysis, this was independent of ICAs and age. For relatives with two or more of these autoantibodies, the risk of diabetes within 3 years was 39% (95% CI, 27–52) and the risk within 5 years was 68% (95% CI, 52–84). Relatives with all three autoantibodies had a risk within 5 years estimated to be 100%. The presence of low first-phase insulin release further increased the risk for relatives with one or two autoantibodies. We conclude that the presence of two or more autoantibodies (out of IAAs, GAAs, and ICA512bdcAAs) is highly predictive of the development of type I diabetes among relatives. *Diabetes* **45:926–933, 1996**

Cytoplasmic islet cell antibodies (ICAs), measured by indirect immunofluorescence on sections of normal human pancreas, are predictive of type I (autoimmune) diabetes in first-degree relatives (1–4). However, this assay is semiquantitative and remains difficult to standardize, despite improvements resulting from standardization workshops (5). Inherent limitations of the ICA assay include the need for subjective scoring of the sections for positivity and wide variation in the results obtained with pancreatic tissue from different donors (6), despite standardization of the results in Juvenile Diabetes Foundation (JDF) units. Recently, several autoantigens involved in type I diabetes have been identified, including ICA512/IA-2, which has homology with the protein tyrosine phosphatase family. The predicted ICA512 molecule described by Rabin et al. (7) is contained within the sequence of the IA-2 molecule described by Lan et al. (8). In the current study, our ICA512/IA-2 assay uses a new construct (termed ICA512bdc) consisting of amino acid residues 256 through 979, incorporating the intracellular domain. This compares with amino acid residues 389 through 937 for the originally described ICA512 and residues 1 through 979 for the full-length IA-2 molecule. Our assay using ICA512bdc gives improved sensitivity, detecting as positive 64% of 50 relatives in whom diabetes subsequently developed, compared with 48% for an assay using ICA512 amino acid residues 389 through 948 (9). In this study, we assessed the predictive value of radioassays for autoantibodies against three islet molecules (insulin [10], GAD [11], and ICA512/IA-2) in a large series of prospectively followed relatives. Using these radioassays in combination, we found that the presence of two or more of insulin, GAD, or ICA512bdc autoantibodies (ICA512bdcAAs) is highly predictive for the development of type I diabetes. These radioassays may be considered as an alternative to the ICA assay, and they are suitable for screening large populations.

RESEARCH DESIGN AND METHODS

Subjects. We studied 882 first-degree relatives of patients with type I diabetes (128 tested at the Joslin Diabetes Center and 754 at the Barbara Davis Center) and 208 healthy general-population control subjects with no family history of diabetes (198 of whom had sufficient serum volume

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FPIR, first-phase insulin release; GAA, GAD autoantibody; IAA, insulin autoantibody; ICA, islet cell antibody; ICA512bdcAA, ICA512bdc autoantibody; IVGTT, intravenous glucose tolerance test; JDF, Juvenile Diabetes Foundation; ROC, receiver operating characteristic.

TABLE 1
The number of subjects testing positive for autoantibodies measured by radioassay (IAAs, GAAs, and ICA512bdcAAs), compared with cytoplasmic ICAs ≥ 20 JDF U, measured by indirect immunofluorescence

					Number of autoantibodies by radioassay (IAAs, GAAs, ICA512bdcAAs)					
	n	IAAs	GAAs	ICA512bdcAAs	1	2	3	≥1	≥2	ICA
Control subjects	198	2 (1.0)	1 (0.5)	0	3 (1.5)	0	0	3 (1.5)	0	3 (1.5)
All first subject-degree relatives	882	107 (12)	150 (17)	64 (7.3)	97 (11)	43 (4.9)	46 (5.2)	186 (21)	89 (10)	119 (14)
Developing diabetes	50	38 (76)	45 (90)	32 (64)	9 (18)	14 (28)	26 (52)	49 (98)	40 (80)	37 (74)
Not developing diabetes	832	69 (8.3)	105 (13)	32 (3.8)	88 (11)	29 (3.5)	20 (2.4)	137 (17)	49 (5.9)	82 (9.9)
BDC1 cohort	683	46 (6.7)	73 (11)	20 (2.9)	68 (10)	19 (2.8)	11 (1.6)	98 (14)	30 (4.4)	48 (7.0)
Remainder: not BDC1 cohort	199	61 (31)	77 (39)	44 (22)	29 (15)	24 (12)	35 (18)	88 (44)	59 (30)	71 (36)
BDC1 cohort of relatives subdivided by relationship to the diabetic proband	683									
Offspring of diabetic father	150	17 (11)*	19 (13)†	8 (5.3)‡	21 (14)	7 (4.7)	3 (2.0)	31 (21)	10 (6.7)	13 (8.7)
Offspring of diabetic mother	137	1 (0.7)	7 (5.1)	0	6 (4.4)	1 (0.7)	0	7 (5.1)	1 (0.7)	6 (4.4)
Sibling	303	21 (6.9)	38 (13)	10 (3.3)	32 (11)	8 (2.6)	7 (2.3)	47 (16)	15 (5.0)	20 (6.6)
Father	35	5 (14)	4 (11)	1 (2.9)	5 (14)	1 (2.9)	1 (2.9)	7 (20)	2 (5.7)	2 (5.7)
Mother	58	2 (3.4)	5 (8.6)	1 (1.7)	4 (6.9)	2 (3.4)	0	6 (10)	2 (3.4)	7 (12)
BDC1 cohort of relatives subdivided by age	683									
<15 years	395	35 (8.9)§	52 (13)¶	15 (3.8)	46 (12)	13 (3.3)	10 (2.5)	69 (18)	23 (5.8)	28 (7.1)
≥15 years	287	11 (3.8)	21 (7.3)	5 (1.7)	25 (7.7)	6 (2.1)	1 (0.3)	29 (10)	7 (2.4)	19 (6.6)

Data are *n* (%). The BDC1 cohort is a subgroup including all relatives sequentially screened for the first time between September 1992 and December 1993 at the Barbara Davis Center. *P = 0.0001, †P = 0.03, ‡P = 0.008, compared with offspring of diabetic mothers. §P = 0.01 and ¶P = 0.02, compared with relatives aged ≥15 years.

to allow testing for all three radioassays and for ICA). The healthy control sera were obtained from laboratory and hospital workers or their children (n = 24), from the Nichols Institute (n = 94), from well children attending an immunization clinic (n = 28), or from another study (12) on the variation of intravenous glucose tolerance test (IVGTT) results in healthy children from the general population (n = 62). The median age of the relatives was 13 years (range, 0.1–76.8 years) and that of the control subjects was 16.2 years (range, 0.4–67.5 years). Relatives were contacted by telephone or mail to determine whether they had developed diabetes. Among 763 relatives who could be traced to determine follow-up status, the median length of follow-up from the date of the serum sample tested was 2.0 years (maximum 11.3).

The relatives studied included three groups. The first was a group of 683 individuals who were unselected for ICAs, consisting of all relatives sequentially screened for the first time between September 1992 and December 1993 at the Barbara Davis Center (this group is referred to as the BDC1 cohort). Secondly, an additional 71 relatives already known to be ICA^+ were identified by screening ~10,000 relatives, mostly at the Joslin Diabetes Center but also at the Barbara Davis Center before September 1992. The third group consisted of 128 ICA^- relatives (also from the Joslin series or studied at the Barbara Davis Center before September 1992) including 10 individuals in whom diabetes developed during follow-up and 118 individuals from a 1-year period for whom follow-up information was obtainable. The latter two groups had similar age distribution: median 11.6 years (range 2.5–66.4 years) for the 71 ICA^+ relatives compared with 12.8 years (range 2.1–69 years) for the 128 ICA^- relatives. The unselected BDC1 cohort had a shorter follow-up (median 2.0 years; maximum 2.6 years) and lower overall frequency of autoantibodies (Table 1) than the other relatives. Five of the relatives developing diabetes came from the BDC1 cohort, compared with 45 from the remainder. In both the Joslin and Barbara Davis Centers, autoantibody-positive relatives are evaluated every 6 months to 1 year with an IVGTT. If the insulin release to intravenous glucose is less than the first percentile of healthy control subjects, the individual is also evaluated with an oral glucose tolerance test to determine whether diabetes is present by National Diabetes Data Group criteria (13).

After 1987, ICA^+ relatives had the opportunity to participate in a trial evaluating the ability of insulin to prevent the onset of diabetes (14). Twenty relatives included in the present study received insulin as part of this prevention trial. Overall, 50 relatives developed type I diabetes during follow-up, including 5 who received insulin in the prevention trial before the onset of diabetes. In survival analyses, the follow-up of these subjects was ended at the time of entry into the trial, although they were included in the analysis of the frequency of autoantibodies among relatives subsequently developing diabetes. The median age at onset was 12.4 years (range 3.9–69 years). The onset of diabetes was defined by the onset of symptoms with hyperglycemia or diabetic oral glucose tolerance test results according to National Diabetes Data Group criteria (13). All subjects or their parents gave informed consent to be studied, and the protocol was approved by the Institutional Review Boards of the University of Colorado and the Joslin Diabetes Center.

Autoantibody assays. Serum samples were stored at −20°C before testing. For each of the 882 relatives, the earliest available serum sample with sufficient volume to allow the measurement of all antibodies was selected. ICAs were measured by indirect immunofluorescence on frozen sections of normal human pancreas (15,16), and titers ≥20 JDF U were considered positive. Our assay achieved ratings of 100% for sensitivity, specificity, validity, and consistency when compared with the 1994 Proficiency Test reference laboratory as a standard (University of Florida, Gainesville, FL). Insulin autoantibodies (IAAs) were measured by a fluid-phase radioassay incorporating competition with cold insulin and precipitation with polyethylene glycol (17). The interassay coefficient of variation for the IAA assay is 10.3% at low positive levels (17). GAAs were measured in triplicate by radioassay, using in vitro transcribed and translated recombinant human GAD (65-kDa isoform) and precipitation with protein A–sepharose (18). The interassay coefficient of variation in our laboratory is 6.5% (n = 10). ICA512bdcAAs were measured in duplicate using a similar assay format but with in vitro transcribed and translated ICA512bdc. The interassay coefficient of variation is 11.7% (n = 9). ICA512bdc is a new construct (E. Kawasaki, unpublished observations) that includes amino acid residues 256 through 979 (compared with amino acids 389 through 937 for the originally described ICA512 and amino acids 1 through 979 for the full-length IA-2 molecule). The results of the GAD autoantibody (GAA) and ICA512bdcAA assays are expressed as an index calculated from the counts per minute for the test sample and the positive and negative control samples (18).

The upper limits of the normal ranges for IAAs (42 nU/ml) and GAAs (index of 0.032) were established as the 99th percentile of the levels in 205 healthy control subjects. We also constructed receiver operating characteristic (ROC) curves (19) for each assay by plotting the true-positive rate (among the 50 relatives in whom diabetes developed) against the false-positive rate (among the control subjects). For ICA512bdcAAs, this revealed that increasing the cutoff from the 99th

percentile in control subjects (index of 0.048) to 0.071 increases specificity from 99 to 100%, with no loss of sensitivity. The ROC curves for IAAs and GAAs supported the use of 99th percentile cutoffs for these assays. For ICAs, using ≥20 JDF U as the criterion for positivity resulted in a false-positive rate of 1.5% among 205 control subjects tested, compared with a true-positive rate (sensitivity) of 74% among the relatives subsequently developing diabetes. Reducing this criterion to ≥10 JDF U increased the false-positive rate to 3.9% with no gain in sensitivity (74%). We therefore considered ≥20 JDF U to be the optimal criterion for ICA positivity.

IVGTTs. One or more IVGTTs were performed on 139 autoantibody-positive relatives. The first-phase insulin release (FPIR) was calculated as the sum of the insulin levels at 1 and 3 min after the end of the glucose infusion. The first percentile for FPIR determined in 225 control subjects is 48 mU/l, and the 10th percentile is 81 mU/l (20). For analysis, data were categorized as >81, 48 through 81, or <48 mU/l. Before March 1990, the IVGTT was performed using 0.5 g glucose/kg body wt. as a 20–25% solution, administered by intravenous infusion over a 2- to 6-min period. For obese individuals (>120% of ideal body weight), 19 g glucose/m^2 body surface area was administered. After March 1990, the protocol was modified slightly to conform with the ICARUS protocol (21), in which 0.5 g glucose/kg body wt. as a 25% solution, up to a maximum of 35 g glucose, is injected over a 3-min period (± 15 s). FPIR data were included in the survival analyses only if the IVGTT was performed within 60 days of the serum sample tested for autoantibodies. Such data were available for 100 relatives, 84 of whom had the IVGTT performed on the same day as the serum sample.

HLA typing. HLA-DQA1 and DQB1 alleles were typed using polymerase chain reaction and sequence-specific oligonucleotide probes (22,23) for 118 relatives, including 28 who were negative for all autoantibodies.

Statistical analysis. The χ^2 was used for comparison of proportions, unless the expected frequency in any cell was <5, in which case Fisher's exact test was used. Survival analysis (product-limit method) was used to estimate the risk of diabetes. Follow-up started with the date of the serum sample tested and ended with the last contact with the subject, entry into the treatment arm of the insulin prevention trial, or the onset of type I diabetes, whichever came first. Subjects lost to follow-up after screening could not be included in survival analyses. Survival curves were compared using the log-rank statistic. The positive predictive value of each test was estimated from the risk of diabetes, separately after 3 and 5 years of follow-up, and 95% CIs around the risk estimates were determined using the variance calculated with Greenwood's formula. The sensitivity of each marker or combination of markers was estimated from the proportion positive among relatives who subsequently developed diabetes; 95% CIs were calculated by normal approximation. Multivariate survival analysis using the Cox proportional hazards model was used to determine the independent contributions of ICAs, age (categorized as <15 and ≥ 15 years), IAAs, GAAs, and ICA512bdcAAs to prediction. Regression models did not include FPIR and HLA because the data were incomplete for these risk markers. Forward stepwise Cox regression (in which each variable is entered into the model, starting with the most statistically significant and ending when all remaining variables are nonsignificant if entered into the model) was used to determine the best model. This model was then extended by the inclusion of remaining variables to evaluate their importance as potential confounders according to their effect on the relative risk (hazard ratio) associated with the main predictor variables.

RESULTS

Frequency of autoantibodies

Specificity in control subjects. The prevalence of autoantibodies in control subjects, prediabetic relatives followed to diabetes, and relatives who have not developed diabetes during current follow-up is compared in Table 1. Results for IAAs, GAAs, and ICA512bdcAAs were each positive in ≤1% of the 198 control subjects tested for all three radioassays and for ICAs. However, none of the control subjects had a positive result for more than one of these autoantibodies, indicating that the presence of two or more of IAAs, GAAs, and ICA512bdcAAs is highly specific. In comparison, 1.5% of the control subjects were ICA ≥20 JDF U (and none of these was positive for another autoantibody).

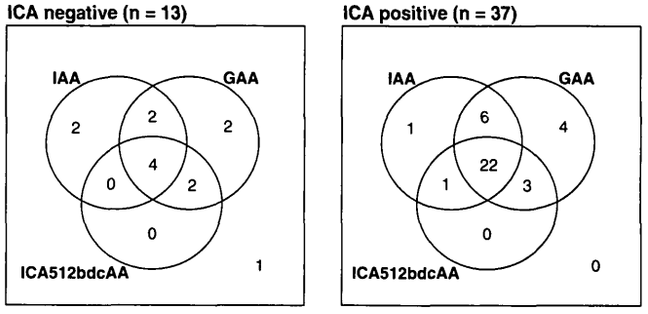

FIG. 1. The frequency of autoantibodies measured by radioassay (IAAs, GAAs, and ICA512bdcAAs) in 50 relatives who developed diabetes during follow-up, according to ICA status. Intersecting regions indicate the number of relatives positive for different combinations of autoantibodies.

Sensitivity in prediabetic relatives. Among 50 relatives who developed diabetes (including 5 who did so after enrollment in the insulin intervention trial), 76% had positive results for IAA, 90% for GAAs, 64% for ICA512bdcAAs, 98% for one or more of these autoantibodies (IAAs, GAAs, or ICA512bdcAAs), 80% for two or more of these, and 74% for ICAs (Table 1). Figure 1 shows the frequency of the autoantibodies measured by radioassay in these prediabetic relatives, according to ICA status. Only one prediabetic relative had negative results for all three of IAAs, GAAs, and ICA512bdcAAs, and this individual also had negative results for ICA. There was no significant difference in the frequency of IAAs, GAAs, and ICA512bdcAAs, comparing ICA^+ and ICA^- prediabetic relatives. The six prediabetic relatives with positive results only for GAAs among the autoantibodies measured by radioassay were older at the onset of diabetes (median 35.4 years) than the other prediabetic relatives (median 11.6 years) ($P = 0.005$, Wilcoxon test).

Co-occurrence of autoantibodies. Among all relatives tested, the autoantibodies occurred together more often than expected by chance. The frequencies of IAAs, GAAs, and ICA512bdcAAs among ICA^+ relatives was 48, 63, and 40%, respectively, compared with 6.6, 9.8, and 2.2% among ICA^- relatives ($P < 0.001$ for each comparison). Tests for GAAs were positive in 71% of IAA^+ vs. 9.6% of IAA^- relatives ($P < 0.001$) and for ICA512bdcAAs were positive in 46% of IAA^+ vs. 1.9% of IAA^- relatives ($P < 0.001$) and in 37% of GAA^+ vs. 1.1% of GAA^- relatives ($P < 0.001$).

Associations between HLA and autoantibodies. When the frequencies of the high-risk haplotypes DR3-DQ2 (DQA1*0501-DQB1*0201) and DR4-DQ8 (DQA1*0301-DQB1*0302) were analyzed for all HLA-typed relatives, ICA512bdcAAs and IAAs both showed an association with DR4-DQ8. DR4-DQ8 was carried by 77% (30 of 39) of $ICA512bdcAA^+$ compared with 43% (34 of 79) of $ICA512bdcAA^-$ relatives ($P = 0.0005$) and by 69% (43 of 62) of IAA^+ compared with 38% (21 of 56) of IAA^- relatives ($P = 0.0005$). These associations were also significant when the analysis was restricted to relatives who did not develop diabetes during follow-up. There was no significant association between either ICA512bdcAAs or IAAs and DR3-DQ2. Neither DR4-DQ8 nor DR3-DQ2 occurred with significantly increased frequency in GAA^+ compared with GAA^- relatives. DR4-DQ8 was carried by 59% (41 of 70) of GAA^+ compared with 48% (23 of 48) of GAA^- relatives ($P = 0.26$), while DR3-DQ2 was carried by 51% (36 of 70) of GAA^+ compared with 44% (21 of 48) of GAA^- relatives ($P = 0.41$).

TABLE 2
Sensitivity and positive predictive value of markers in first-degree relatives of patients with type I diabetes: cytoplasmic ICA, autoantibodies measured by radioassay (IAAs, GAAs, and ICA512bdcAAs) and FPIR

		Positive predictive value	
	Sensitivity	Risk by 3 years	Risk by 5 years
IAAs	76 (64–88)	33 (21–44)	59 (41–78)
GAAs	90 (81–98)	28 (19–37)	52 (38–66)
ICA512bdcAAs	64 (51–77)	40 (26–54)	81 (64–98)
ICAs (≥20 JDF U)	74 (62–86)	31 (20–43)	51 (35–67)
GAAs and IAAs	68 (55–81)	41 (27–55)	68 (50–86)
GAAs and ICA512bdcAAs	62 (49–76)	45 (30–60)	86 (71–100)
IAAs and ICA512bdcAAs	54 (40–68)	47 (31–65)	100
GAAs or IAAs	98 (94–100)	25 (17–33)	48 (34–61)
GAAs or ICA512bdcAAs	92 (85–100)	27 (18–36)	50 (36–64)
IAAs or ICA512bdcAAs	86 (76–96)	31 (0–41)	58 (42–74)
Number of autoantibodies by radioassay (IAAs, GAAs, and ICA512bdcAAs)			
1	18 (7.4–29)	8.0 (1.6–15)	15 (0.9–28)
2	28 (16–40)	30 (12–49)	44 (21–66)
3	52 (38–66)	49 (32–66)	100
≥1	98 (94–100)	24 (16–32)	46 (33–60)
≥2	80 (69–91)	39 (27–52)	68 (52–84)
0 abs by radioassay and ICAs	0	0	0
≥1 abs by radioassay and ICAs	74 (62–86)	41 (28–54)	59 (44–74)
≥2 abs by radioassay and ICAs	64 (51–77)	47 (31–63)	79 (61–97)
≥1 abs by radioassay and FPIR >81 mU/l	18 (5.9–30)	16 (2.9–28)	21 (5.3–36)
≥1 abs by radioassay and FPIR 48–81 mU/l	28 (14–42)	22 (1.8–43)	66 (36–96)
≥1 abs by radioassay and FPIR <48 mU/l	51 (36–67)	79 (61–97)	100
≥2 abs by radioassay and FPIR >81 mU/l	15 (4.1–27)	19 (2.0–36)	29 (5.4–53)
≥2 abs by radioassay and FPIR 48–81 mU/l	21 (7.8–33)	13 (0–29)	82 (50–100)
≥2 abs by radioassay and FPIR <48 mU/l	41 (26–57)	87 (70–100)	100

Data are % (95% CI). Positive predictive value (after 3 and 5 years of follow-up) was determined by survival analysis on 763 relatives screened for autoantibodies and for whom follow-up information was available (100 with FPIR data). Sensitivity was determined as the proportion with the marker among 50 relatives in whom diabetes developed during follow-up (39 with FPIR data). abs, autoantibodies.

Eleven relatives carried the protective HLA allele DQB1*0602 (24), and none developed diabetes during follow-up ranging from 0.7 to 11.3 years. Of these, five had negative results for all autoantibodies, one had positive results for ICAs alone, three for GAAs and ICAs, one for IAAs and GAAs, and one for all autoantibodies. Among 36 prediabetic relatives (subsequently developing diabetes) with HLA typing data available, 33% had DQ2/DQ8 (DR3/DR4), 19% had DQ2/DQX (where DQX is any haplotype other than DQ8), 33% had DQ8/DQX (where DQX is any haplotype other than DQ2), and 14% had neither DQ2 nor DQ8.

Frequency of autoantibodies according to relationship to the proband and age. The frequency of each of the antibodies was lower in the BDC1 cohort than in the other relatives studied (Table 1), because this group of sequentially screened relatives was unselected for ICAs. When antibody frequencies in this group were analyzed according to the relationship to the diabetic proband, the offspring of diabetic fathers had a higher frequency of IAAs ($P = 0.0001$), GAAs ($P = 0.03$), and ICA512bdcAAs ($P = 0.008$) than the offspring of diabetic mothers (Table 1). IAAs and GAAs were also more frequent in younger relatives, aged <15 years ($P = 0.01$ and 0.02, respectively).

Predictive value of markers

Individual autoantibodies, age and HLA type. Using survival analysis to allow for different lengths of follow-up for the relatives, the presence of IAAs, GAAs, ICA512bdcAAs, or ICA was each associated with increased risk of diabetes ($P < 0.0001$ in each case, log-rank test). The risk of diabetes (positive predictive value) within 5 years was 81% for ICA512bdcAAs, 59% for IAAs, 52% for GAAs, and 51% for ICAs (Table 2). There was also a significant increase in risk associated with age <15 years at the time of screening compared with age of ≥15 years ($P = 0.02$). The presence of the HLA-DQB1*0602 allele was protective from development of diabetes ($P = 0.03$). None of 11 relatives carrying this allele developed diabetes; follow-up ranged from 0.7 to 11.3 years. Conversely, HLA-DQ2/DQ8 (DR3/DR4) heterozygosity significantly increased the risk of diabetes ($P = 0.003$).

Autoantibodies in combination. Estimates of the positive predictive value (estimated by survival analysis) and sensitivity for different combinations of risk markers are listed in Table 2; specificity can be estimated from the data for control subjects in Table 1. There was a progressive increase in risk according to the number of autoantibodies measured by radioassay (IAAs, GAAs, or ICA512bdcAAs) among all relatives, comparing zero, one, two, and three autoantibodies ($P < 0.0001$, log-rank test, Fig. 2*A*). For relatives with two or more of these autoantibodies, the risk of diabetes within 3 years was 39% (95% CI, 27 through 52) and the risk within 5 years was 68% (95% CI, 52 through 84). For relatives with all three of these autoantibodies, the estimated risk within 5 years was 100%, significantly increased versus two autoantibodies ($P = 0.002$).

Among 97 relatives positive for only a single autoantibody measured by radioassay, GAA was the most frequent ($n = 64$, 66%), followed by IAA ($n = 28$, 29%) and ICA512bdcAA ($n = 5$, 5%). Subdividing the group positive for a single radioassay autoantibody by the type or level of autoantibody present did not significantly improve prediction.

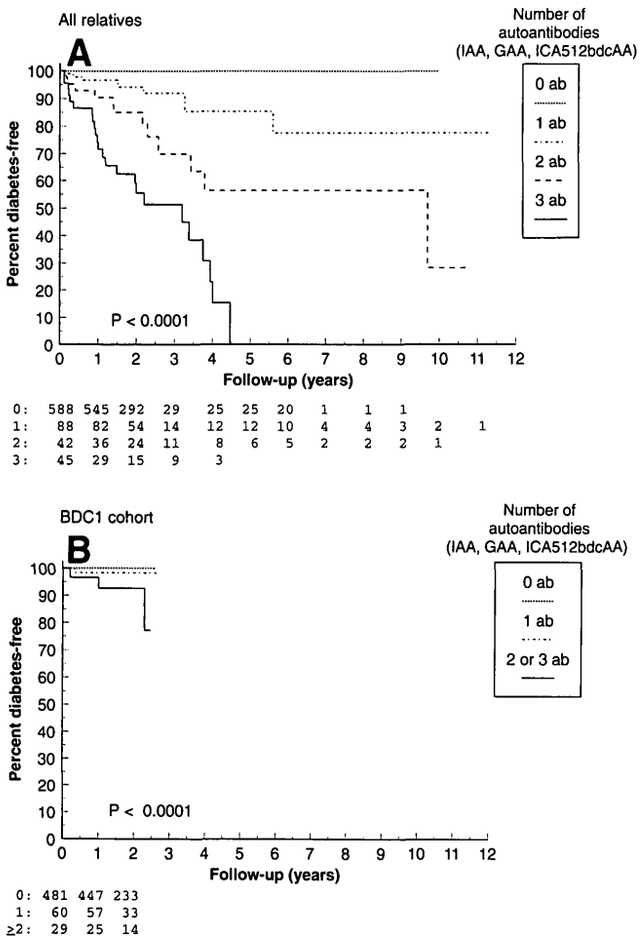

FIG. 2. The diabetes-free survival of first-degree relatives, according to the number of autoantibodies (ab) present at baseline, considering IAAs, GAAs, and ICA512bdcAAs. *A:* data for all relatives tested. *B:* data for a subgroup of sequential first-degree relatives screened for the first time (BDC1 cohort).

The number of autoantibodies (IAAs, GAAs, and ICA512bdcAAs) was significantly predictive within both ICA^+ ($P < 0.0001$) and ICA^- ($P < 0.0001$) subgroups of relatives. Conversely, when the relatives were stratified by the number of autoantibodies measured by radioassay (one, two, or three), the additional presence of ICAs did not significantly increase the risk of diabetes ($P = 0.06$, 0.17, and 0.30, respectively). The progressive increase in risk according to the number of autoantibodies positive by radioassay was also true in the BDC1 cohort of sequential relatives screened for the first time ($P < 0.0001$, Fig. 2*B*).

The results of multivariate analysis using Cox regression in the 763 relatives for whom follow-up information was available are listed in Table 3. This analysis shows that neither ICA nor age made an independent contribution to prediction after allowing for the autoantibodies measured by radioassay. Model 1 in Table 3 was determined by forward stepwise Cox regression with the variables ICAs, age (categorized as <15 and ≥15 years), IAAs, GAAs, and ICA512bdcAAs. Neither ICAs nor age was statistically significant, and both were therefore dropped from the final model. Model 2 was determined in similar fashion with the variables ICAs, age, and number of autoantibodies positive by radioassay (counting IAAs, GAAs, and ICA512bdcAAs). Again, neither ICAs nor age made a statistically significant contribution. Within each model, the relative risk for each variable is adjusted for the other variables in that model. For example, the independent effect of positivity for GAAs is a 16-fold increase in the relative risk of diabetes, after allowing for the effects of ICA512bdcAAs and IAAs (model 1 in Table 3). Although ICAs and age were not statistically significant in either model, we considered that they should be evaluated as potential confounding factors. However, as shown in models 1a and 2a of Table 3, the addition of ICAs and age to model 1 or model 2 made no meaningful change to the relative risks associated with the radioassay autoantibodies, indicating that ICAs and age are not important confounding factors. Similar results were obtained with models in which ICAs or age were added individually.

TABLE 3
Independent relative risks for development of type I diabetes in 763 relatives

Risk Factor	Relative risk	*P* value
Model 1: specific autoantibody type		
GAAs	16.1 (4.3–59.4)	<0.001
ICA512bdcAAs	3.5 (1.8–7.0)	<0.001
IAAs	2.6 (1.2–5.5)	0.013
Model 1a: addition of ICA and age to Model 1		
GAAs	14.1 (3.7–53.5)	<0.001
ICA512bdcAAs	3.0 (1.4–6.6)	0.006
IAAs	2.7 (1.2–6.1)	0.01
ICAs	1.5 (0.67–3.4)	0.31
Age <15 years	1.3 (0.64–2.7)	0.46
Model 2: number of radioassay autoantibodies		
Number of ab:		
0	1.0	
1	39.8 (5.0–319)	<0.001
2	114.9 (14.8–893)	<0.001
3	352.6 (47.5–2617)	<0.001
Model 2a: addition of ICA and age to Model 2		
Number of ab		
0	1.0	
1	33.7 (4.1–277)	0.001
2	106.5 (13.1–866)	<0.001
3	267.4 (31.8–2252)	<0.001
ICA	1.6 (0.72–3.4)	0.25
Age <15 years	1.3 (0.65–2.8)	0.42

Data are relative risk (95% CI). Calculations were done with Cox regression models. Within each model, the relative risk for each variable is adjusted for the other variables in that model. Models 1 and 2 were determined by forward stepwise Cox regression and retain only statistically significant variables. Neither ICAs nor age make a significant independent contribution to prediction in either model. In models 1a and 2a, ICAs and age are added but make no meaningful change to the relative risks associated with the radioassay autoantibodies, indicating that ICAs and age are not important confounding factors. ab represents autoantibodies measured by radioassay (GAAs, ICA512AAs, and IAAs). Reference category: risks for 1, 2, or 3 autoantibodies are risks compared with 0 autoantibodies. For models 1a and 2a, relative risks for age <15 years are compared with age ≥15 years.

Combination of FPIR and autoantibodies. The presence of low FPIR increased the risk of diabetes in both the subgroup positive for a single autoantibody measured by radioassay ($P < 0.006$) and the subgroup positive for multiple autoantibodies measured by radioassay ($P < 0.0001$) (Fig. 3). In both subgroups, the additional presence of FPIR

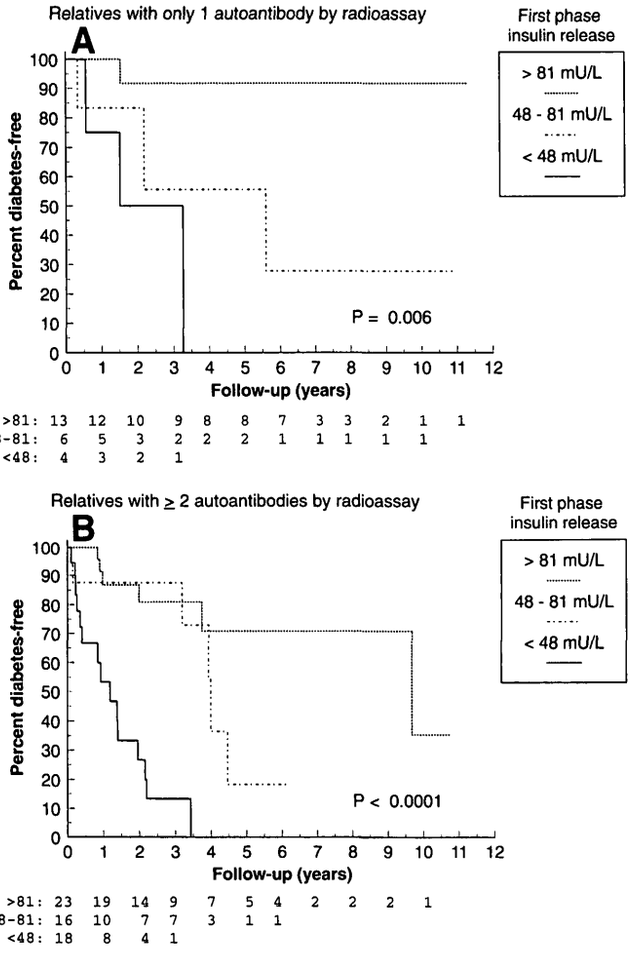

FIG. 3. The diabetes-free survival of first-degree relatives, according to the FPIR measured by IVGTT within 60 days of the serum sample tested for autoantibodies. *A:* data for relatives with only one of the autoantibodies measured by radioassay (IAAs, GAAs, or ICA512bdcAAs). *B:* data for relatives with two or more of these autoantibodies.

below the first percentile of normal control subjects (48 mU/l) was associated with an estimated risk of diabetes of 100% within 4 years. Among relatives with preserved FPIR at baseline (greater than the 10th percentile of control subjects, 81 mU/l), those with positive results for multiple autoantibodies measured by radioassay had a high risk of diabetes with extended follow-up, whereas those with positive results for only one of these autoantibodies had a low risk (Fig. 3).

DISCUSSION

We found that the presence of two or more autoantibodies measured by radioassay (IAAs, GAAs, or ICA512bdcAAs) is highly predictive of diabetes risk in first-degree relatives, independent of age and ICAs, and that prediction can be further improved by the measurement of FPIR. We estimate that the presence of one or more of these autoantibodies has 46% positive predictive value with 5 years of follow-up, 98% sensitivity, and 98.5% specificity, whereas the presence of two or more has 68% positive predictive value, 80% sensitivity, and 100% specificity. The presence of all three radioassay autoantibodies gives 100% positive predictive value, 52% sensitivity, and 100% specificity. In comparison, the figures for ICA (≥20 JDF U) are 51% positive predictive value, 74% sensitivity, and 98.5% specificity.

The combination of low FPIR and multiple autoantibodies measured by radioassay markedly increased positive predictive value, although this was at the expense of reduced sensitivity (Table 2) if only a single IVGTT at baseline is considered. The FPIR is an indirect measure of the remaining β-cell mass and declines with progress toward the onset of diabetes. Repeated measurement of FPIR in autoantibody-positive relatives is therefore likely to increase the effective sensitivity of this test.

Bingley et al. (25) found that additional testing of ICA^+ relatives for IAAs, GAAs, and antibodies against a 37-kDa islet antigen improved the predictive value of ICAs alone. Bonifacio et al. (26) found evidence that the 37-kDa antigen is a fragment of IA-2. Our findings further suggest that the combined use of IAAs, GAAs, and ICA512bdcAAs is highly predictive, irrespective of ICA status. A shortcoming of our study is that 71 of the relatives were selected for ICAs, although we included a sample of 128 ICA^- relatives from the same series and 683 of the relatives (the BDC1 cohort) were unselected for ICAs. Furthermore, the number of autoantibodies (out of IAAs, GAAs, and ICA512bdcAAs) was predictive within both ICA^- and ICA^+ subgroups of the relatives and within the unselected BDC1 cohort, despite relatively short follow-up in this group. In multivariate analyses, the additional presence of ICAs did not significantly increase the risk of diabetes after allowing for the number of autoantibodies positive by radioassay. Although this does not exclude an additional contribution of ICAs detectable in a larger data set or with a different ICA assay, the information in Tables 2 and 3 suggests that the use of the three radioassays in combination is a satisfactory alternative to the older ICA assay by immunohistochemistry. The radioassays are quantitative and have the important practical advantage of being semiautomated. The GAA and ICA512bdcAA assays can be performed using only 7 μl of serum in a 96-well plate format. It should be possible to screen large populations initially for the two most easily measured autoantibodies, GAAs and ICA512bdcAAs (together detecting 92% of prediabetic relatives), followed by the measurement of IAAs in subjects positive for one or both of these.

Within the unselected BDC1 cohort, 4.4% of relatives were positive for two or more of the autoantibodies measured by radioassay, similar to the risk of diabetes in first-degree relatives (~6%). However, the frequency of one or more of these autoantibodies (14.3%) may be higher than the risk of diabetes, indicating that some relatives may express a single autoantibody without developing diabetes. We also found that the offspring of diabetic fathers are more likely to express autoantibodies than the offspring of diabetic mothers, with significantly higher frequencies of IAAs, GAAs, and ICA512bdcAAs. This is consistent with the reported increase in the risk of diabetes for the offspring of diabetic fathers (27).

We found a significant association of both ICA512bdcAAs and IAAs with the presence of the HLA-DR4-DQ8 haplotype. This haplotype is also associated with type I diabetes, but these associations remained significant if relatives subsequently developing diabetes were excluded. An association of IAAs with DR4 also has been reported in new-onset type I diabetic subjects (28). Serjeantson et al. (29) and Hagopian et al. (30) both found an association between GAAs and the HLA-DR3-DQ2 haplotype in new-onset (30) or recent-onset (29) type I diabetic patients. Although there was a similar

trend among the relatives that we studied, this was not statistically significant.

A positive ICA reaction may indicate the presence of antibodies directed against one or more β-cell antigens. The simultaneous detection of multiple autoantibodies in this way may account for the high predictive value of ICAs as a single test. GAD has been shown to be one of the antigens that may be recognized by antibodies in the ICA test. The ICA reactivity of a minority of ICA^+ subjects can be abolished by preabsorption of the serum with GAD (31,32). These subjects, with restricted ICAs that react only with GAD, have a low risk of diabetes (33). Thus, GAAs may form one component of ICAs, but 10 of 45 (22%) GAA^+ relatives who developed diabetes were ICA^- in the present study, indicating that the ICA assay does not detect all GAA^+ subjects. Other antigens recognized by ICA remain to be identified. Likely candidates include ICA512/IA-2 (26) and a glycolipid molecule (34). The identification of other β-cell autoantigens, whether or not they are recognized in the ICA assay, is likely to further improve prediction using the combined autoantibody approach. It is also possible that relatives destined to develop diabetes may express increasing numbers of autoantibodies as they progress closer to the onset of diabetes, reflecting the loss of tolerance to an increasing range of β-cell autoantigens.

We conclude that the presence of two or more autoantibodies (out of IAAs, GAAs, and ICA512bdcAAs) is highly predictive for the development of type I diabetes among relatives. We recommend that intervention trials include these markers in the assessment of diabetes risk in relatives.

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