Alistair J.K. Williams,[1] Vito Lampasona,[2] Rebecca Wyatt,[1] Cristina Brigatti,[3] Kathleen M. Gillespie,[1] Polly J. Bingley,[1] and Peter Achenbach[4]

Reactivity to N-Terminally Truncated GAD_{65}(96–585) Identifies GAD Autoantibodies That Are More Closely Associated With Diabetes Progression in Relatives of Patients With Type 1 Diabetes

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GAD autoantibodies (GADAs) identify individuals at increased risk of developing type 1 diabetes, but many people currently found to be GADA positive are unlikely to progress to clinical disease. More specific GADA assays are therefore needed. Recent international workshops have shown that the reactivity of sera from healthy donors varies according to assay type and indicated that the use of N-terminally truncated GAD_{65} radiolabels in GADA radiobinding assays is associated with higher specificity. To determine whether a radiobinding assay using radiolabeled GAD_{65}(96–585) identified individuals who are at higher risk of developing diabetes, samples from recent-onset patients and GADA-positive first-degree relatives participating in the Bart's-Oxford type 1 diabetes family study were reassayed with full-length or N-terminally truncated GAD using the National Institute of Diabetes and Digestive and Kidney Diseases harmonized protocol. The sensitivity in patients was the same with both labels, but fewer relatives retested positive with truncated GAD. Among relatives who progressed to diabetes, similar proportions were found to be GADA positive when tested with either label, but because of their higher specificity the cumulative risk of diabetes was higher in those with autoantibodies to GAD_{65}(96–585). Autoantibodies to GAD_{65}(96–585) in relatives are more closely associated with diabetes risk than those to full-length GAD, suggesting that assays using N-terminally truncated GAD should be used to select participants for intervention trials.

GAD autoantibodies (GADAs) are the most widely used marker of type 1 diabetes. They are a mainstay of diabetes prediction and recruitment to therapeutic intervention trials, as well as being used for disease characterization (1). However, many individuals found to be GADA positive with current assays are unlikely to progress to type 1 diabetes. Improved discrimination of diabetes risk can be achieved by testing for multiple islet autoantibodies, but the development of more disease-specific GADA assays that enable more efficient screening for type 1 diabetes is a high priority (2).

Recent international islet autoantibody workshops revealed systematic differences in reactivity between ELISAs and radiobinding assays (RBAs), which suggested that the performance of many RBAs may be improved by the use of the N-terminally truncated radiolabel ^{35}S-GAD_{65}(96–585) (3). We therefore assessed the ability of an RBA using the truncated GAD label to identify patients with recent-onset type 1 diabetes and to discriminate diabetes progression in first-degree relatives (FDRs) of type 1 diabetes patients in comparison with an assay using full-length GAD_{65}.

[1]School of Clinical Sciences, University of Bristol, Bristol, U.K.
[2]Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milan, Italy
[3]Diabetes Research Institute, San Raffaele Scientific Institute, Milan, Italy
[4]Institute of Diabetes Research, Helmholtz Zentrum München, and Forschergruppe Diabetes, Klinikum rechts der Isar, Technische Universität München, Neuherberg, Germany

Corresponding author: Alistair J.K. Williams, a.j.k.williams@bristol.ac.uk.

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RESEARCH DESIGN AND METHODS

Patients With Recent-Onset Type 1 Diabetes

To determine the diabetes sensitivity of autoantibodies to the different GAD constructs, sera from 147 patients (89 male and 58 female, median age 11.6 years, age range 1.3–21 years) with recent-onset type 1 diabetes (median diabetes duration 1 day, range −7 to 90 days) were randomly selected from a well-characterized cohort (4), and assayed for GADA(1–585) and GADA(96–585).

FDRs of Type 1 Diabetes Patients Previously Screened for GADAs

Sera were available from 283 relatives (139 male and 144 female) who had previously been found to be GADA positive with a local RBA using ^{35}S-labeled full-length GAD_{65}, after the screening of 4,470 FDRs (2,121 male and 2,349 female) in the Bart's-Oxford (BOX) family study (5). These GADA-positive relatives were followed up prospectively for disease development by an annual questionnaire (median follow-up to last contact or diabetes diagnosis 14 years, range 0.2–27.7 years, median age 31.4 years, age range 1.3–57.4 years). A subset of 459 of the first available samples (from 227 male and 232 female FDRs) was randomly selected from the 4,187 BOX FDRs who previously had screened negative with the local GADA assay and reassayed for GADA(1–585) and GADA(96–585). This subset was enriched with 31 of 179 screen-negative relatives who developed diabetes (Table 1). Local RBAs were used to measure autoantibodies to IA-2 (IA-2A) and insulin (IAA) (5) on all relatives, and zinc transporter 8 (ZnT8A) (6) on those relatives who initially had positive screening results for GADAs or who subsequently developed diabetes.

Autoantibody Assays

Sera from patients and relatives were assayed for GADAs using the National Institute of Diabetes and Digestive and Kidney Diseases harmonized assay protocol (7) with ^{35}S-methionine–labeled antigens made by in vitro transcription and translation of both N-terminally truncated GAD_{65}(96–585) and full-length GAD_{65} encoded in the pTNT plasmid vector (Promega, Madison, WI) (3). To localize N-terminal epitopes more precisely, a subset of samples from the FDRs who had positive screening results was also reassayed for GADAs using ^{35}S-labeled GAD_{65}(46–585). A methionine residue was added to both truncated antigens to allow transcription. Samples were considered to be positive if they had autoantibody levels ≥97.5th percentile of 222 healthy schoolchildren (8); equivalent to 13.5 digestive and kidney (DK) units/mL for GADA (1–585), 12.8 DK units/mL for GADA(96–585), and 25.4 DK units/mL for GADA(46–585). Using these thresholds, the sensitivity of the GADA(1–585) assay was 72% at a specificity of 92%, and the sensitivity of the GADA (96–585) assay was 70% at 99% specificity in the Islet Autoantibody Standardization Program 2013 workshop. The interassay coefficient of variation of GADA(1–585) was 12.9% at 53 DK units/mL (n = 32). The interassay coefficient of variation of GADA(96–585) was 13.0% at 59 DK units/mL (n = 32).

HLA Genotyping

HLA class II genotyping was available on 209 of the 283 (74%) GADA-positive FDRs. HLA class II *DRB1*, *DQA1*, and *DQB1* analysis was performed on blood and mouth swab DNA with sequence-specific primers, as previously described (9). Haplotypes were established based on common patterns of linkage disequilibrium.

Data Analysis

Categorical variables were compared using the χ^2 test. Genetic risk was analyzed according to the high-risk haplotypes *DRB1*03-DQA1*0501-DQB1*0201* (*DR3-DQ2*) and *DRB1*04-DQA1*0301-DQB1*0302* (*DR4-DQ8*), as well as the protective haplotype *DRB1*02-DQB1*0602* (*DR2-DQ6*). Other haplotypes were classified as X. Survival analysis was performed using the Kaplan-Meier method, and the Mantel-Cox log-rank test was used to compare survival between groups. For all analyses, a two-tailed P value of 0.05 was considered significant. The area under the

Table 1—Characteristics of FDRs participating in the BOX family study whose samples were originally screened for GADAs using a local assay and were reassayed using the standard method with ^{35}S-labeled GAD_{65}(1–585) and GAD_{65}(96–585)

	GADA positive		GADA negative	
	Nonprogressors (n = 213)	Progressors (n = 70)	Nonprogressors (n = 428)	Progressors (n = 31)
Male	102	37	204	23
Median age, years (range)	31.5 (1.3–57.4)	30.9 (1.6–52.9)	32.7 (1.7–57.3)	39 (1.4–56)
Median age at diagnosis, years (range)		38 (3.2–69.8)		52 (3.3–68.5)
Median follow-up, years (range)	14.9 (0.6–27.7)	7.2 (0.2–24.2)	15.2 (0–27.6)	12.3 (1.2–23.8)
Additional autoantibodies*	37	39	11†	4
IA-2A	13	22	0	2
IAA	23	23	11	3
ZnT8A	19	27		1

Data are number of FDRs or median (range). *Additional autoantibodies are from IA-2A, IAA, and ZnT8A. †Not assayed for ZnT8A.

curve (AUC) of the receiver operating characteristic (ROC) with 95% CI was calculated assuming a nonparametric distribution of results using R software. Other statistical analyses were performed using the Statistics Package for Social Sciences Version 21 (IBM, New York, NY).

RESULTS

Patients With Recent-Onset Type 1 Diabetes

Using thresholds equivalent to the 97.5th percentile in schoolchildren, the sensitivity of GADA(96–585) was identical to GADA(1–585) in patients with recent-onset type 1 diabetes (Fig. 1*A*). Of 147 patients, 117 (80%) were positive for GADA(96–585), and 117 were positive for GADA(1–585). One hundred sixteen patients (79%) had autoantibodies to both GAD constructs, and there was excellent correlation between the levels of GADA (96–585) and GADA(1–585) (r = 0.99, $P < 0.001$; Fig. 2*A*). The ROC-AUCs based on the patients and schoolchildren were also very similar for GADA(1–585) and GADA(96–585) (0.94 [95% CI 0.91–0.97] and 0.93 [95% CI 0.9–0.96], respectively) (Fig. 3).

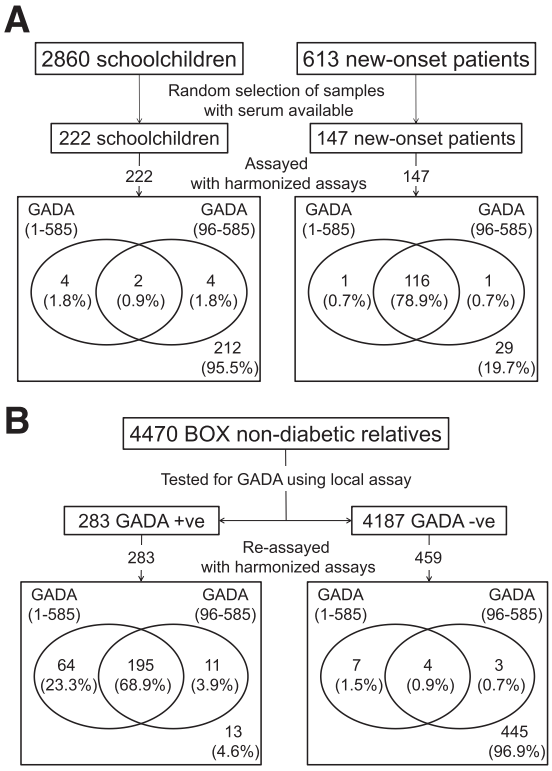

Figure 1—Scheme showing the selection of samples, and results for healthy schoolchildren and patients with newly diagnosed type 1 diabetes (*A*) or BOX relatives (*B*), following reassay with the harmonized protocol using either ^{35}S-labeled GAD$_{65}$(1–585) or GAD$_{65}$(96–585). The same proportion of patients was found to be positive for GADA(96–585) as for GADA(1–585). However, fewer relatives previously found to be GADA positive with the local assay were found to be positive on reassay for GADA(96–585) rather than for GADA(1–585) ($P < 0.001$). −ve, negative; +ve, positive.

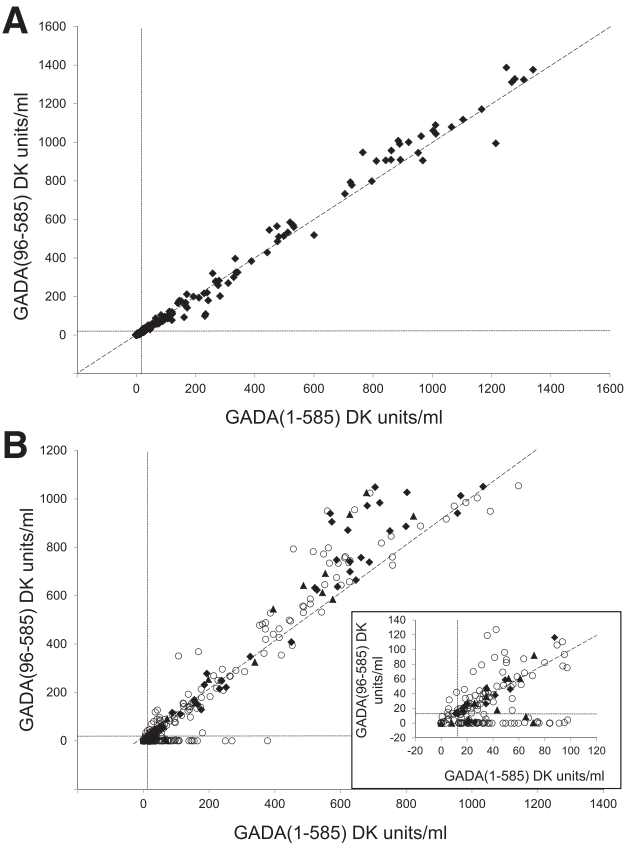

Figure 2—Plot of GADA(96–585) against GADA(1–585) results for 147 patients with recent-onset type 1 diabetes (*A*) and 283 FDRs (*B*) from the BOX family study who previously screened positive for GADAs with a local RBA. The inset is an expanded plot of results for samples with GADA(1–585) levels up to 100 DK units/mL. Relatives who progressed to diabetes within 10 years are indicated with closed diamonds, those whose conditions progressed after 10 years are indicated with closed triangles, and relatives whose conditions did not progress are indicated by open circles. Thresholds for the assays are given by the dotted lines, and equivalent levels of both autoantibodies are indicated by the dashed lines. Correlation of GADA(96–585) with GADA(1–585) was excellent for patients (y = 1.04 * x − 4.79, R = 0.99) and very good for FDRs (y = 1.16 * x − 14.84, R = 0.96), although many sera from FDRs found to be positive for GADA(1–585) with levels up to 378 DK units/mL were found to be negative for GADA(96–585).

Relatives Who Previously Screened GADA Positive

Of 283 relatives who were previously found to be positive for GADAs using the local RBA, 259 (92%) were positive on reassay using the harmonized assay protocol with ^{35}S-GAD$_{65}$(1–585), 206 (73%) were positive with ^{35}S-GAD$_{65}$(96–585), and 195 (69%) were positive with both labels (Fig. 1*B*). Of 70 relatives who subsequently developed diabetes, 66 (94%) were positive for GADA (1–585), 63 (90%) were positive for GADA(96–585), and 61 (87%) were positive for both specificities. Of 76 relatives previously found to be positive for GADAs who had at least one additional islet autoantibody, 73 (96%) were positive for GADA(1–585), 70 (92%) were positive for GADA(96–585), and 69 were positive for both specificities.

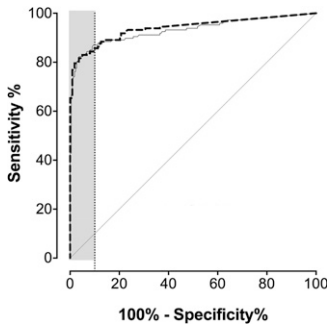

Figure 3—ROC-AUCs for GADA(1–585) (bold dashed line) and GADA(96–585) (solid line) based on data from 147 patients with newly diagnosed type 1 diabetes and 222 healthy schoolchildren. The autoantibodies performed similarly; the AUC was 0.94 for GADA(1–585) and 0.93 for GADA(96–585) (P = 0.28). The partial AUC calculates areas at specificities >90% (within the gray box), and was 0.081 for GADA(1–585) and 0.080 for GADA(96–585) (P = 0.69).

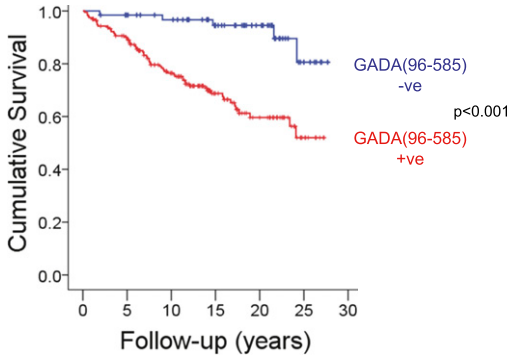

Figure 4—A Kaplan-Meier survival curve for FDRs positive for GADA(1–585) according to positivity for GADA(96–585). GADA (96–585) identified FDRs at increased risk of diabetes progression; few FDRs who were positive for GADA(1–585), but negative for GADA(96–585), developed diabetes within 20 years of follow-up. −ve, negative; +ve, positive.

Of these multiple antibody-positive relatives, diabetes developed in 39 (53%) with GADA(1–585) and 38 (54%) with GADA(96–585). Of 207 relatives with no additional autoantibodies, 187 (90%) were positive for GADA(1–585) compared with 136 (66%) who were positive for GADA(96–585) ($P < 0.001$). There was a good correlation between the levels of GADA(1–585) and GADA(96–585) in sera from the GADA-positive relatives (r = 0.96, $P < 0.001$) (Fig. 2*b*). Of 64 relatives positive for GADA (1–585), but negative for GADA(96–585), 38 (59%) had GADA(1–585) levels of <50 DK units/mL, a level found in 27 of the 147 (18%) recent-onset patients. The deletion of amino acids 46 to 95 of GAD65 was important in improving specificity with little loss of sensitivity; of the 64 samples with GADA(1–585) alone, 49 (77%) were positive for GADA(46–585), of whom diabetes developed in only 4 (8%).

Kaplan-Meier survival analysis showed that positivity for GADA(96–585) identified relatives positive for full-length GADAs who were at increased risk of diabetes progression (Fig. 4; $P < 0.001$). Of the 11 relatives who rescreened positive only for GADA(96–585), 1 had additional autoantibodies (IAA and IA-2A), but was lost to follow-up after 4 years, while diabetes developed in 2 others after 5 and 6 years of follow-up. Of 160 relatives carrying at least one HLA risk haplotype who rescreened positive for GADA(1–585), 129 (81%) were positive for GADA(96–585), compared with 18 of 35 (51%) with no HLA risk haplotype ($P < 0.001$). Furthermore, positive screening results for GADA(96–585) were less common in GADA-positive relatives carrying protective haplotypes; of 13 relatives carrying *HLA-DQ6*, 12 were positive for GADA(1–585), but only 3 were positive for GADA (96–585) (P = 0.001).

Relatives Who Previously Screened GADA Negative

Of the 428 relatives who remained nondiabetic during follow-up who had previously screened negative with the original GADA assay, 7 (1.6%) were positive for GADA(1–585) alone, 2 (0.5%) were positive for GADA(96–585) alone, and 4 (0.9%) were positive for both. Of the 31 relatives whose conditions progressed to diabetes, but who screened negative for GADAs with the original assay, none were positive for GADA(1–585) and 1 was positive for GADA(96–585).

DISCUSSION

GADA measured using N-terminally truncated antigen achieved the same sensitivity in recent-onset patients as the assay using full-length GAD_{65}, while those relatives having autoantibodies to GAD_{65}(96–585) were at higher risk of disease progression than those with autoantibodies to full-length GAD alone. Survival analysis showed that very few GADA-positive relatives without autoantibodies to GAD_{65}(96–585) developed diabetes within 20 years. Furthermore, only a minority of GADA-positive relatives who carried protective HLA haplotypes were found to be positive when using the N-terminally truncated label.

Birth cohort studies (10,11) of relatives of type 1 diabetes patients have shown that autoantibody epitope reactivity typically spreads from the COOH-terminal and middle (pyridoxal phosphate binding) regions to the N-terminal domains of the molecule. Autoantibodies to the N-terminal region normally constitute a relatively minor component of GAD autoreactivity and in isolation have little association with progression to diabetes (12). Our data would support this observation, since most samples from relatives who developed diabetes showed similar antibody binding and levels with full-length ^{35}S-GAD_{65} and ^{35}S-GAD_{65}(96–585) radiolabels (Fig. 2*B*). Furthermore, the majority (59%) of relatives found to be positive for GADA(1–585), but negative for GADA(96–585), had relatively low levels of GADA (<50 DK units/mL), which is consistent with a less vigorous autoimmune response in

these individuals. This explains why sensitivity in recent-onset patients was maintained, but most relatives carrying protective haplotypes and only a small proportion of relatives with multiple islet autoantibodies were found to be negative when GADAs were measured using the truncated construct.

Several groups have investigated the effect of N-terminal truncations of GAD_{65} on the disease sensitivity of GADA. Deletion of the first 194 amino acids did not cause decreased binding by eight prediabetic/diabetic sera (13), while in agreement with our findings an assay using ^{125}I-labeled GAD_{65}(46–585) performed similarly to an assay using ^{35}S-labeled full-length GAD_{65} (14). However, to our knowledge, this is the first study to show improved discrimination of diabetes progression using an N-terminally truncated GAD_{65} label. The use of truncated antigens is established practice for the measurement of autoantibodies to IA-2 and ZnT8, since the main diabetes-relevant epitopes are located in the intracellular portion of IA-2 and the carboxy terminal region of ZnT8 (15,16). If confirmed in other populations, including young children, our finding suggests that screening strategies to identify individuals at high risk of developing diabetes should use GADA RBAs based on N-terminally truncated protein.

Although fewer autoantibodies to disease-irrelevant GAD_{65} epitopes were detected using the N-terminally truncated label, the Kaplan-Meier survival curve suggests that diabetes will develop in fewer than half of GADA(96–585)–positive relatives within 25 years (Fig. 4). N-terminally truncated GADAs were associated with autoantibodies to other islet antigens, but could not discriminate the risk of progression within multiple antibody-positive relatives. Further improvements in assay specificity are therefore desirable. This may be achieved by more radical N-terminal deletions, if additional diabetes-irrelevant epitopes are disrupted without affecting binding to diabetes-relevant epitopes. Our addition of an N-terminal methionine to GADA (96–585) to allow protein expression is unlikely to have affected antibody binding as it is neither highly charged nor bulky. The inclusion of affinity measurements may also help to identify GADA-positive individuals who are at increased risk of diabetes progression (12,17). The potential for truncated GAD_{65} labels to identify patients with slow-onset autoimmune diabetes in adults with a clinical presentation of type 2 diabetes also needs to be investigated. A previous study (18) using GAD_{65}/GAD_{67} chimeras rather than truncated GAD_{65} found no difference in the time to insulin requirement between those patients with or without N-terminal autoantibodies.

RBAs are still widely used for the prediction and characterization of type 1 diabetes despite the advent of high-quality alternative assay formats such as the bridging ELISA (19) and electrochemiluminescence assay (20). Advantages of RBAs include their relatively low cost, high sensitivity, good flexibility, small serum volume requirement, and proven track record in diabetes prediction as well as the wide availability of equipment and reagents. However, a major shortcoming of GADA RBAs has been their relative lack of specificity. We have demonstrated that use of an N-terminally truncated GAD_{65} label can improve the disease specificity of the GADA assay without the loss of sensitivity in patients and can identify GADA-positive relatives who are at higher risk of disease progression. As the recruitment of high-risk relatives to therapeutic intervention trials normally includes initial testing for GADAs, these findings strongly suggest that the adoption of autoantibody assays using N-terminally truncated GAD_{65} would greatly improve screening efficiency for future studies aimed at preventing type 1 diabetes.

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