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Risk for developing Type 1 (insulin-dependent) diabetes mellitus and the presence of islet 64K antibodies

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Summary. First-degree relatives of Type 1 (insulin-dependent) diabetic patients are at increased risk for developing clinical diabetes. The presence of islet cell or insulin autoantibodies further identifies relatives at greater risk, but not all immunologic-marker-positive relatives progress to disease. Beta-cell dysfunction, however, seems to be more prevalent than clinical Type 1 diabetes, since stable subclinical pancreatic Beta-cell dysfunction may occur. Antibodies against a M_r 64,000 (64K) islet Beta-cell protein, identified as glutamic acid decarboxylase, have been reported both at and several years prior to the clinical onset of Type 1 diabetes. We measured 64K antibodies in first-degree relatives with varying degrees of Beta-cell dysfunction and risk for subsequent Type 1 diabetes to determine whether 64K antibodies improve the predictive power of islet cell antibodies and/or insulin autoantibodies. In the Seattle Family Study first-degree relatives of Type 1 diabetic patients are followed prospectively using detailed Beta-cell function tests, insulin sensitivity, quantitative evaluation of islet cell antibodies and fluid phase assay insulin autoantibodies. 64K antibodies were measured using dog islets. Relatives were selected, based on Beta-cell function to represent individuals at high ($n = 6$) and low ($n = 30$) risk for subsequent Type 1 diabetes. The 30 low-risk individuals followed-up for 78 months, had stable Beta-cell function, and six (20%) were negative for all autoantibodies, ten (33%) were positive for insulin autoantibodies, 16 (53%) were islet cell antibody positive while six (20%) were positive for 64K antibodies. In contrast, of the six subjects with progressively declining Beta-cell function who are therefore at high risk, two of whom have already developed Type 1 diabetes, two (33%) were positive for insulin autoantibodies, four (67%) were islet cell antibody positive, while all six (100%) were positive for 64K antibodies. We conclude that antibodies to the M_r 64,000 islet protein correlate with progressive Beta-cell dysfunction more closely than either islet cell antibodies or insulin autoantibodies, but can sometimes be present in individuals whose Beta-cell function remains stable over several years.

Key words: Type 1 (insulin-dependent) diabetes mellitus, insulin autoantibodies, islet cell antibodies, glutamic acid decarboxylase antibodies, intravenous glucose tolerance test, prediabetes.

An immunological pathogenesis for Type 1 (insulin-dependent) diabetes mellitus is supported by the presence of inflammatory cells in the islets of Langerhans at onset [1–3] and of autoantibodies, reacting with pancreatic islet cells [4–6], insulin [7], or a Beta-cell M_r 64,000 (64K) protein [8], as early as several years prior to clinical onset [9–12].

Prediction of future Type 1 diabetes, however, has been hampered by low sensitivity and specificity of conventional risk markers, such as islet cell antibodies (ICA), insulin autoantibodies (IAA) or certain human leucocyte antigen (HLA) specificities. A better predictive value may be obtained if several markers are combined [13, 14].

Since 64K antibodies are detected at a high frequency in new onset Type 1 diabetic patients [13, 15, 16] and may precede clinical onset by several years [12, 13], we addressed their relation to other markers in individuals at risk for Type 1 diabetes. Recently, evidence was presented that the 64K antigen is glutamic acid decarboxylase (GAD) [17] present mostly in brain cells but also in pancreatic Beta cells [18]. The islet 64K antigen is detected by immunoprecipitation of metabolically labelled islet preparations of a variety of different species [8, 15, 19]. In the present study, we analysed 64K antibodies in a quantitative assay using dog islets.

We asked whether 64K antibodies could distinguish between subgroups of first-degree relatives of Type 1 diabetic patients followed in the Seattle Family Study [20]. Four groups of first-degree relatives were defined based primarily on whether pancreatic Beta-cell function

Table 1. Clinical information on first-degree relatives

	Age	Sex	Relationship to proband	Discordance (years)	Follow-up (months)	IAA %	ICA	JDF units	64K antibodies
Group 1									
1	31	m	HLA-identical sibling	12	62	–	–	0	–
2	37	f	HLA-identical sibling	21	71	–	–	0	–
3	36	f	HLA-identical sibling	10	57	–	–	0	–
4	40	f	HLA-identical sibling	21	63	–	–	0	–
5	31	f	HLA-identical sibling	21	39	–	–	0	–
6	32	f	HLA-identical sibling	12	74	–	–	0	–
Group 2									
7	47	f	Sibling	47	28	1.0	–	0	–
8	46	f	Haploidentical sibling	25	78	1.5	–	0	–
9	32	f	Parent	32	22	2.8	–	0	–
10	11	m	Sibling	6	23	4.2	–	0	–
11	18	f	Parent	18	5	2.3	–	0	–
12	31	f	Child	4	15	2.5	–	0	–
13	35	m	Parent	2	2	1.8	–	0	–
14	13	m	Child and sibling	13	1	3.5	–	0	–
Group 3									
15	45	f	HLA-identical sibling	34	70		+/–	80	–
16	40	f	Haplo-identical sibling	37	60	–	+	40	–
17	37	m	Haplo-identical sibling	13	78	–	+/–	20	–
18	27	f	Haplo-identical sibling	15	58	–	+/–	320	–
19	37	m	Haplo-identical sibling	13	50	–	+/–	80	–
20	58	m	Parent	24	46	–	+/–	160	–
21	23	f	HLA-identical sibling	17	72	–	+/–	20	+
22	15	m	Sibling	3	29	–	+	160	+
23	49	f	Parent	23	50	2.0	+	40	+
24	51	f	Parent	10	16	–	+/–	40	–
25	34	m	Sibling	5	6	–	+	180	+
26	38	f	Parent	12	27	–	+	40	+
27	35	f	Parent	6	27	–	+	80	–
28	16	m	Sibling/child	3	26	2.1	+	40	+
29	13	m	Sibling	3	22	–	+/–	40	–
30	35	m	Sibling	?	7	–	+/–	20	–
Group 4									
31	23	f	Identical twin	3	15	–	–	0	+
32	25	f	Sibling	18	5	–	–	0	+
33	36	m	Sibling	16	39	–	+/–	20	+
34	18	f	Identical twin	3	27	2.5	+	80	+
35	21	m	Sibling (2)/child	13	20	6.3	+	320	+
36	15	f	Sibling	15	17	–	+	80	+

The insulin autoantibody (IAA) value (% binding) shown is at the last follow-up. Islet cell antibodies (ICA) are indicated as negative (–), positive (+) or transient (+/–). The highest ICA levels are shown in Juvenile Diabetes Foundation (JDF) units and 64K antibodies as positive (+) or negative (–)

was stable or was progressively declining. Those with stable Beta-cell function were subdivided, depending on the presence or absence of IAA or ICA. The relationship of 64K antibodies to these subgroups was then determined.

Subjects and methods

Subjects

All 36 subjects were first-degree relatives of Type 1 diabetic probands and were part of the prospective Seattle Family Study [20]. They were divided based on their pancreatic Beta-cell function (methodology described in detail below) into two main groups. The first had stable Beta-cell function ($n = 30$) since their acute insulin response to glucose (AIRgluc) was greater than 50% of that expected at entry to the study, at all subsequent timepoints, and declined by less than 15% during follow-up. These subjects were further divided into HLA identical siblings who were negative for both ICA and IAA (Group 1, $n = 6$), relatives who were IAA positive only (Group 2, $n = 8$) and those who were ICA positive (Group 3, $n = 16$). Relatives with progressively impaired Beta-cell function (Group 4, $n = 6$) had AIRgluc less than 50% of that expected either at entry or at all subsequent timepoints, and showed decline of over 20% during follow-up. Clinical information on all subjects is given in Table 1. Beta-cell function (first and last timepoints) is shown in Figure 1.

Beta-cell function tests

Tests of Beta-cell function were carried out on two separate days for each subject. The methodology has been described in detail elsewhere [20, 21]. Briefly, following an overnight fast a modified

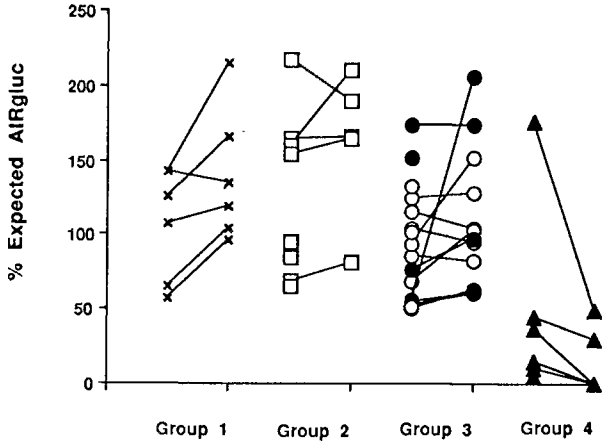

Fig. 1. Beta-cell function data from Groups 1–4. The % expected acute insulin reponse to intravenous glucose (AIR_{gluc}) at entry and at the most recent timepoint of the study are plotted for each group. Crosses depict Group 1, squares Group 2; circles Group 3, and triangles Group 4. Follow-up times were, for Group 1 39–74 months, Group 2 1–78 months, Group 3 6–78 months and Group 4 5–39 months. Filled symbols identify subjects who are 64K-antibody positive

intravenous glucose tolerance test (IVGTT) was performed to determine the acute insulin response to intravenous glucose (AIG_{gluc}) calculated as the mean rise above baseline in circulating insulin during the first 10 min following i.v. glucose. Bergman's minimal model [22, 23] was applied to obtain the insulin sensitivity index (Si). The inverse relationship between Si and AIR_{gluc} among a normal non-diabetic population [21] allows us to express each subject's actual AIR_{gluc} as a percentage of the expected value (% expected AIR_{gluc}) based on insulin sensitivity. This allows interpretation of Beta-cell function over time in relation to changes in insulin sensitivity. The mean percentage expected AIR_{gluc} for non-diabetic individuals is 100. Among a group of 18 non-diabetic control subjects studied on two occasions separated by an average of 13 months, the intra-individual coefficient of variation for expected AIR_{gluc} was 10.5%.

64K antibody analysis

Dog islets were isolated as previously detailed [24, 25]. Metabolic labelling was done in batches of 10,000–15,000 dog islets for 6 h at 37°C in L-methionine free RPMI 1640 with 0.5% (volume/volume) newborn calf serum (Gibco, Grand Island, N.Y. USA), 20 mmol/l glucose, 20 mmol/l Hepes, 24 mmol/l $NaHCO_3$, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) supplemented with 1 mCi ^{35}S-methionine (Amersham Inc., Arlington Heights, Ill., USA). Islets were homogenized in 10 mmol/l Hepes (pH 7.4) containing 0.25 mol/l sucrose, 0.5% (weight/volume) aprotinin (Novo, Bagsvaerd, Denmark), 10 mmol/l benzamidine-HCl and 0.1 mmol/l p-chloromercuribenzenesulphonic acid per batch of islets, followed by centrifugation at 12,000 × *g* for 30 min. The pellet was extracted using 10 mmol/l Hepes (pH 7.4), 150 mmol/l NaCl, 10 mmol/l benzamidine-HCl, 0.5% (weight/volume) aprotinin and 2% Triton X-114. Subsequent temperature-dependent phase separation [26] yielded a detergent-rich pellet. To decrease non-specific binding, this pellet was precleared by incubation with normal human serum followed by Protein A-Sepharose (LKB Bio Technology, Piscataway, N.J., USA). The precleared material was used for the Protein A-Sepharose facilitated immunoprecipitation with 15 µl of serum from each individual. A standard 64K antibody-positive serum which is also the Juvenile Diabetes Foundation (JDF) reference serum for ICA detection [27] and a 64K antibody-negative control serum (from a healthy ICA-negative individual) were run in parallel and included on every gel as internal controls. Immune complexes were eluted by boiling and analysed on discontinuous 8% SDS-polyacrylamide gels under reducing conditions [28]. After staining and destaining, the gels were processed for fluorography with Amplify (Amersham). Dried gels were exposed to Kodak X-omat AR films (Kodak Company, Rochester, N.Y., USA) at −80°C.

The 64K antibodies were determined in all 36 individuals by analysing blind the entire set of follow-up samples from every individual on the same gel. The presence or absence of a 64K band was first scored visually on coded fluorograms. Only the positions of the positive or negative reference sera were revealed to the observer. Other bands present on the gels were not scored since they represented non-specific binding (Fig. 2). The fluorograms were therefore also analysed by densitometry using an LKB Ultroscan LX to express the results as a 64K Index [19]. A 64K Index of 0.29 (x + 2 SD of a panel of 20 normal healthy control subjects) was shown to separate visually rated 64K-antibody positive from negative sera. Dilution of the positive control serum showed a linear relationship between the amount of serum used and the 64K Index. The inter-assay coefficient of variation (cv) was 12.3%; the intra-assay cv for duplicate determinations was 5.6%.

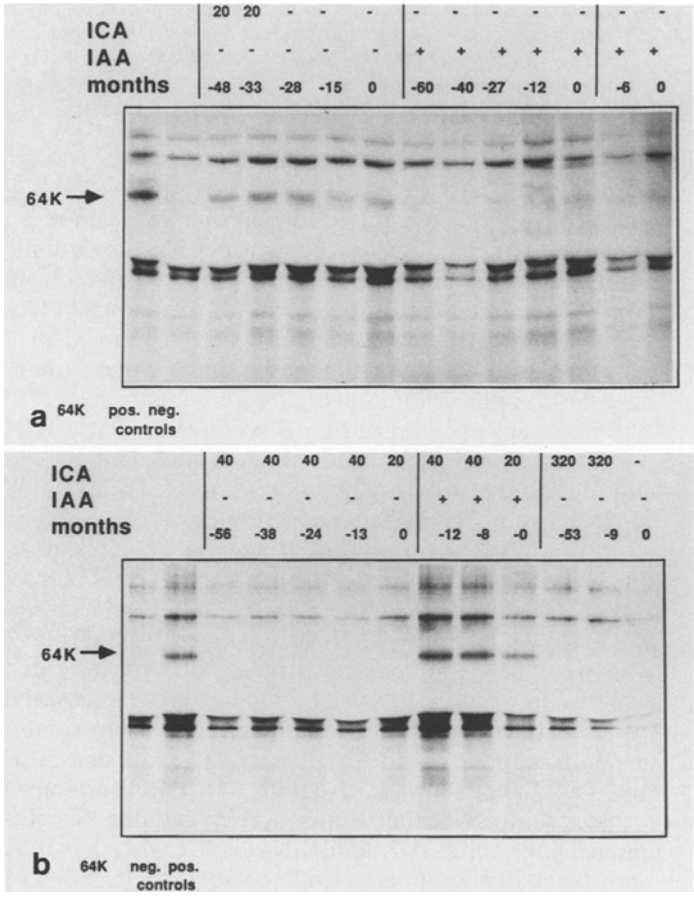

Fig. 2a, b. Fluorograms of immunoprecipitations to detect 64K-antibody positive sera. In panel A the follow-up samples from one Group 3 sibling (relative 21 in Table 1) (far left) is shown together with two relatives (8 and 9, respectively) from Group 2. The most recent timepoint (0 months) is shown on the right. Islet cell antibody (ICA) are indicated in Juvenile Diabetes Foundation units if positive. Insulin autoantibodies (IAA) are indicated as positive (+) or negative (−). The 64K-antibody positive and negative controls are shown in the first two lanes. In panel B samples in Group 3 (siblings 16, far left, 28 and 18) are shown. Antibodies against the 64K protein were detected in subjects 21 and 28

Islet cell antibody assay

An indirect immunofluorescence assay was used to determine end-point titres of ICA. All sera were absorbed with rat liver powder. Sections of snap-frozen, blood-group 0 human pancreas were incubated with serum in doubling dilutions. Washing in phosphate-buffered 150 mmol/l NaCl was followed by addition of fluorescein isothiocyanate-conjugated goat anti-human IgG (Sigma, St. Louis, Mo., USA). All samples, including a positive and negative control in each assay, were evaluated blind by two independent observers. ICA were considered to be present if fluorescence was detected by both observers in two separate assays. The results were expressed in Juvenile Diabetes Foundation (JDF) units [27] using the international JDF reference serum as standard. For evaluation of changes in ICA status in the individuals followed prospectively, the end-point titre was determined at all time points for that individual in the same assay. The lower detection limit for our assay was 10 JDF units.

Insulin autoantibody assay

IAA were measured using an RIA as previously described [7, 29] and displacement with cold insulin as stipulated by the International Workshop on Standardization of Insulin Autoantibodies [30]. Our laboratory participates in the International Diabetes Workshop and proficiency-testing programs for both ICA and IAA.

Results

The 64K antibodies in the follow-up samples from every individual were analysed on the same gel. Examples of the gels are shown in Figure 2 a and b. Subjects 8 and 9 from Group 2, persistently positive for IAA, are shown in Figure 2 a. None of the subjects in Groups 1 or 2 were positive for 64K antibodies at any timepoints and are therefore illustrated as open symbols in Figure 3 a.

Seven subjects from Group 3 were persistently ICA positive, of whom two were also IAA positive (subjects 23 and 28) and five were 64K antibody-positive (subjects 22, 23, 25, 26 and 28). Nine subjects from Group 3 were only intermittently ICA positive, of whom none was IAA positive but one was 64K antibody-positive (subject 21). Subject 21 is illustrated in Figure 2 a, while subjects 16, 18 and 28 are shown in Figure 2 b. All six subjects from Group 3 who were 64K antibody-positive are shown as filled symbols in Figure 3 b. 64K antibodies were measured on 23 occasions during follow-up of 6–68 (median 21) months in these six subjects and remained positive on all occasions. Subject 21 had 64K antibodies measured at seven timepoints over 68 months. She remained persistently 64K antibody-positive while her ICA went from 20 JDF units to undetectable levels. None of the subjects in Groups 1–3 have developed Type 1 diabetes.

Of the six subjects in Group 4, three were persistently ICA positive, of whom two were also IAA positive, one was intermittently ICA positive while two were both ICA and IAA negative. However, all six were 64K antibody-positive on at least one timepoint. Subject 31 was 64K antibody-negative on entry to the study but was 64K antibody-positive at her most recent timepoint. Subject 33 has gone from being 64K antibody-positive to negative while

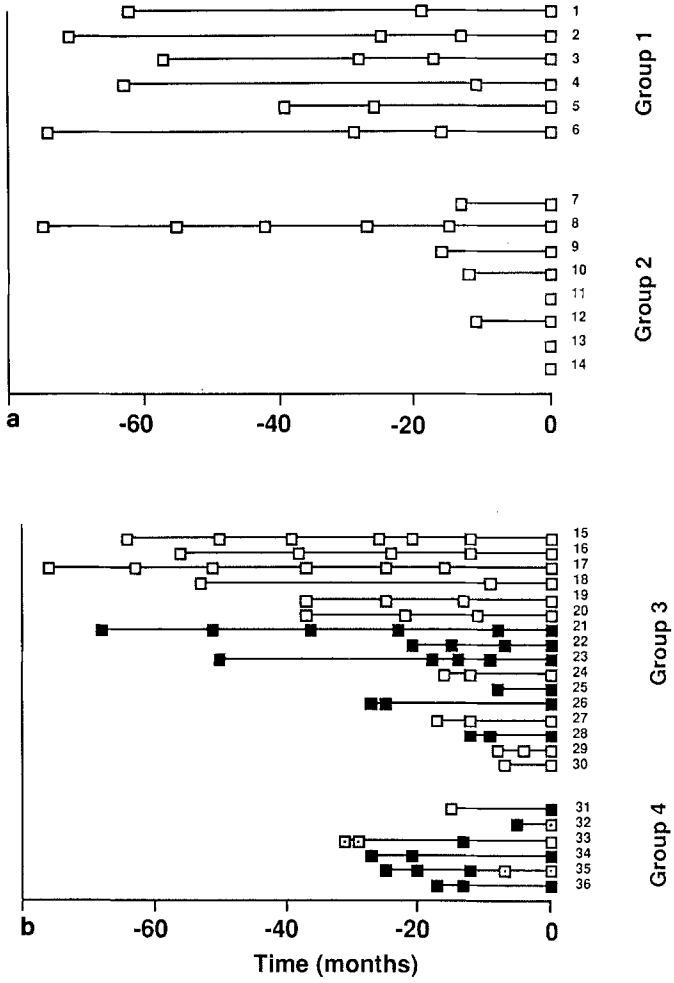

Fig. 3 a, b. Presence of 64K antibodies in first-degree relatives in Groups 1–2 (panel A) and 3–4 (panel B). Individual subjects as detailed in Table 1, are plotted separately. Time in months with the most recent timepoint being 0 months is shown on the horizontal axis. 64K-antibody positive samples are depicted as filled squares, 64K-antibody negative samples as open squares. Timepoints for which 64K antibodies' data are not available are depicted as open squares with a dot in the centre

subjects 32, 34, 35 and 36 have been 64K antibody-positive at all timepoints tested. Subjects 31 and 34 have developed clinical Type 1 diabetes about 12 months after their last timepoint.

Discussion

Detailed follow-up studies in relatives of Type 1 diabetic patients have shown that subclinical states of Beta-cell dysfunction that remain stable can occur among ICA-positive relatives as well as monozygotic twins of Type 1 diabetic patients [20, 34] without progression to clinical diabetes. The spectrum of Beta-cell dysfunction ranging from subtle changes to clinical diabetes may include an unknown percentage of individuals with stable subclinical Beta-cell dysfunction who are not progressing to clinical Type 1 diabetes. It is therefore critical to distinguish individuals with progressively impaired Beta-cell function from individuals with stable Beta-cell function in order to

assess their risk for future clinical Type 1 diabetes. In this study we have subdivided relatives based on the results of prospective Beta-cell function testing and have then examined the antibody status within subgroups. 64K antibodies were present in all subjects with progressively impaired Beta-cell function, including two subjects who were negative for both ICA and IAA. However, 64K antibodies were present in only 20% of those whose Beta-cell function was stable. Thus, the presence of 64K antibodies appears to be a better marker for disease progression than either ICA or IAA. However, the fact that 64K antibodies can be found in some subjects who are at relatively low risk for future clinical Type 1 diabetes is interesting. Three of the subjects in Group 3 who were positive for 64K antibodies over several years (subjects 21, 23 and 26) are adults who have already been discordant for at least 12 years. During follow-up of up to 6 years there is no evidence of progressive decline in Beta-cell function and so the risk of clinical diabetes developing in these individuals is probably low. These data therefore suggest that the presence of 64K antibodies does not always predict subsequent clinical Type 1 diabetes.

It is clear that no single genetic or immunologic marker yet identified can completely distinguish those subjects with progressive from those with stable Beta-cell dysfunction, although many are helpful, especially when found in combination. An increased risk for Type 1 diabetes is associated with certain haplotypes of the HLA class II DR and DQ specificities [31]. However, these specificities, although necessary, are not sufficient since monozygotic twins are only 30–50% concordant for the disease [32–34]. Siblings of a Type 1 diabetic patient have a 16–30% risk of developing clinical Type 1 diabetes by the age of 30 years if they are HLA identical, but have only a 5% risk if they are haploidentical [35–37]. However, due to this genetic risk, family studies following relatives of Type 1 diabetic patients provide a unique opportunity to develop possible risk markers and to investigate their predictive value.

Islet cell antibodies (ICA) have been found in 3–21% of first-degree relatives of Type 1 diabetic patients [35, 38], but also in as many as 2–4% of the general population [39, 40]. ICA-positive individuals therefore outnumber those eventually developing clinical Type 1 diabetes, since the prevalence of Type 1 diabetes in the general population is 0.15–0.3% [41, 42]. Furthermore, ICA levels may fluctuate or be only transiently high [46], and they correlate poorly with Beta-cell function [43]. IAA, even though detectable in some patients before onset are strongly correlated with age but poorly associated with Beta-cell dysfunction or future Type 1 diabetes in ICA-negative individuals [43, 44]. Individuals with not only high titre ICA but also IAA and/or a decrease in first-phase insulin response have been reported to be at higher risk for Type 1 diabetes [14, 45, 46]. A large study on ICA in first-degree relatives showed that ICA-positive relatives in multiplex families are at a higher risk for developing Type 1 diabetes than relatives of simplex families [47]. Our finding that 64K antibodies correlate with progressively impaired Beta-cell function and high risk for Type 1 diabetes suggests that 64K antibodies may be a more specific marker for autoimmune processes leading to Type 1 diabetes than ICA and/or IAA.

The role of 64K antibodies in the disease process of Type 1 diabetes has yet to be clarified. They were detected in selected individuals up to 8 years prior to onset [12] with high frequency at onset [15, 19] and prolonged persistence after onset [48]. The 64K antibodies were also detected in sera from diabetic BB rats [49] and non-obese diabetic (NOD) mice [50] as early as the time of weaning. Thus, 64K antibodies are present early in the pathogenesis not only of human Type 1 diabetes but also in autoimmune diabetic syndromes in rodents.

More recently the finding of 64K antibodies before the onset of clinical Type 1 diabetes was confirmed in a group of 28 pre-diabetic patients [13] also demonstrating the presence of 64K antibodies in first-degree relatives associated with both ICA (≥ 20 JDF units) and IAA, which is in agreement with our data. In contrast to our finding, four of five individuals, who were positive for IAA only, had 64K antibodies. However, they were all younger than 5 years of age and such individuals were not included in our study. IAA are present at higher frequencies in this age group [51, 52]. In newly diagnosed diabetic patients no effect of age on 64K-antibody frequencies was detected in patients aged 0–34 years [15, 19]. Furthermore, in our prospective study we identified individuals with fluctuating or disappearing ICA who, nevertheless, were 64K antibody positive. Thus, 64K antibodies were not restricted to ICA-positive individuals with titres ≥ 20 JDF units [13]. It should be noted that the age of the subjects and length of discordance in Group 4 are not significantly lower than the other groups.

Quantitation of 64K antibodies should eventually aid understanding of the natural history of these antibodies in Type 1 diabetes. In this study we determined 64K-antibody positivity using a quantitative 64K Index. The identification of the 64K protein as glutamic acid decarboxylase [17] should permit further refinement in the quantitative analysis of these antibodies in subjects at risk for developing Type 1 diabetes. The 64K Index [19] is however currently the most powerful way to obtain objective and quantitative 64K-antibody levels. In this assay system a positive and negative reference serum are used to compute the 64K Index based on densitometric scanning of fluorograms, thus minimizing inter-assay variation and allowing analysis of changes in 64K-antibody levels.

It is obviously necessary to be cautious when interpreting prospective data such as those in our study. We cannot say with certainty that the remaining four individuals in Group 4 will develop clinical Type 1 diabetes in the future (although two have already done so), or that none of those in Groups 1, 2 and 3 will do so. However, a prospective evaluation of the end-organ being attacked (the pancreatic Beta-cell) would seem to be the most informative way to evaluate the pre-clinical period. It is clear that Type 1 diabetes is not a disease restricted to childhood, but rather one affecting people of all ages, with wide variation in the spectrum of disease activity and rate of progression. We conclude that 64K antibodies reflect progressively impaired Beta-cell function better than ICA or IAA and therefore are useful in the follow-up of individuals at risk

for Type 1 diabetes. Their measurement, in combination with other clinical data, may eventually lead to the identification of the individuals at highest risk such that preventive treatment may be administered before a significant loss of Beta cells induces insulin dependence and clinical onset of the disease.

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