Original Articles

Antibodies to GAD and Tryptic Fragments of Islet 64K Antigen as Distinct Markers for Development of IDDM

Studies With Identical Twins

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Insulin-dependent diabetes mellitus (IDDM) is associated with antibodies to a 64,000-M_r islet cell protein, at least part of which is identified as glutamic acid decarboxylase (GAD). These antibodies are detected as two distinct antibody specificities to 50,000-M_r and 37,000/40,000-M_r tryptic fragments of the autoantigen (50K and 37K antibodies, respectively). We determined the frequencies of antibodies to intact GAD, tryptic fragments of islet 64,000-M_r antigen, islet cell antibodies (ICAs), and insulin autoantibodies (IAAs) in sera from 58 nondiabetic identical twins of patients with IDDM, of whom 12 subsequently developed diabetes. ICA, antibodies to intact GAD, and those to tryptic fragments were detected at similar frequencies in prediabetic twins (67–75%), but only 25% had IAA. Of 46 twins who remain nondiabetic, GAD antibodies, 50K antibodies, and ICA were detected in 6 (13%), 7 (15%), and 5 (11%), respectively, whereas only 1 (2%) possessed 37K antibodies and 2 (4%) had IAA. Eight of 9 twins with 37K antibodies and all 6 twins with ICA >20 Juvenile Diabetes Foundation U have developed diabetes. Antibodies to GAD are sensitive markers for diabetes development but may also be present in genetically susceptible individuals who are unlikely to develop disease. Antibodies to 37,000/40,000-M_r fragments of the 64,000-M_r antigen or high-titer ICA were the best markers for diabetes development in these twins. *Diabetes* 41:782–87, 1992

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Current evidence suggests that insulin-dependent diabetes mellitus (IDDM) is the result of the specific destruction of pancreatic β-cells by an immune-mediated mechanism (1). The presence of immunological and metabolic changes several years before clinical onset of disease implies a prolonged clinically latent prediabetic period (2,3). Antibodies reacting with islets on frozen sections of human pancreas (islet cell antibodies [ICA]; 2) or with insulin (insulin autoantibodies [IAAs]; 4) can be detected during this period and may be of value in the prediction of disease. Studies of other islet cell components recognized by serum antibodies indicate that antibodies to a 64,000-M_r islet cell protein may also represent an early, sensitive, and specific marker for subsequent development of disease (5–7). The 64,000 M_r islet antigen has been identified as the γ-aminobutyric acid synthesizing enzyme, glutamic acid decarboxylase (GAD), which is also expressed in brain tissue (8). GAD exists as multiple isoforms, two of which (GAD_{65} and GAD_{67}) have been cloned and sequenced (9). Both GAD_{65} and GAD_{67} are expressed in islets, and both forms may be recognized by diabetes-associated antibodies (10–12). GAD enzyme activity has also been detected in other nonneuronal tissues (13), but these GAD isoforms may not be recognized by antibodies in diabetes (14).

Our own studies suggest that there is a heterogeneous antibody response to the 64,000-M_r antigen (15). Limited trypsin digestion of the islet 64,000-M_r antigen yields three major polypeptide products of 50,000, 40,000, and 37,000 M_r. Diabetic patients possess at least two distinct antibody specificities that recognize determinants on these fragments. One specificity (50K antibodies) binds 50,000-M_r fragments; the other (37K antibodies) binds both 37,000- and 40,000-M_r fragments. In most patients, antibodies that recognize the 50,000-M_r tryptic fragment also bind the intact 64,000-M_r antigen (15) and GAD

(14). In contrast, 37K antibodies are a new antibody specificity that does not recognize intact 64,000-M_r antigen or GAD and may bind determinants that are hidden on the native molecule or may be directed against a distinct protein. There is evidence that the 50,000- and 37,000-M_r components of antigen are derived from different polypeptide chains (15,16). More than 90% of children with recent-onset IDDM possess antibodies to tryptic fragments of the 64,000-M_r antigen, and these antibodies are potentially highly sensitive markers for diabetes development (15). However, autoimmunity to the 64,000-M_r antigen complex may not be restricted to individuals at risk for diabetes: A nondiabetic child with juvenile rheumatoid arthritis was also positive for antibodies to the 50,000-M_r tryptic fragment (15). Further studies are required to determine the value of antibodies to the 64,000-M_r antigen as predictive markers for diabetes.

Nondiabetic identical twins of IDDM patients are an important population group for the study of immunological changes in diabetes. Because they are genetically identical to their diabetic twin, these individuals possess disease-susceptibility genes but have a ~30% risk of developing diabetes (17). More than 90% of the individuals in this group that develop diabetes will do so within 6 yr of diabetes diagnosis in their twin (17); thus, the probability of an individual developing disease can be estimated in follow-up studies. In this study, we analyzed serum from 58 nondiabetic twins of IDDM patients for antibodies to intact GAD, to tryptic fragments of the 64,000-M_r antigen, and for ICA and IAA. The twins have been followed after serum sampling for subsequent development of diabetes. These studies have allowed us to compare the value of antibodies to islet cell components as markers for disease.

RESEARCH DESIGN AND METHODS

The nondiabetic twins studied were selected from a cohort of 156 pairs of identical twins, referred as discordant for diabetes from throughout Britain between 1967 and 1989. Monozygosity was established in all twin pairs, as previously described (18). Of the total cohort, 120 nondiabetic twins were still nondiabetic when first seen by us and these twins have been followed prospectively for development of diabetes. Diabetes is diagnosed according to the National Diabetes Data Group criteria (19). Twins were selected for this study *1*) if they had a nondiabetic oral glucose tolerance test within 3 yr of the close of the study on 31 December 1989 (nondiabetic twins) or if they developed diabetes (prediabetic twins) and *2*) if sufficient serum was available for antibody analysis. Fifty-eight twins fulfilled these criteria, and one serum for each twin was selected at random from stored samples. The 58 twins were representative of the cohort from which they were selected in terms of age at onset of the index twin, age at close of the study, sex ratio, and incidence of diabetes. Twelve of the twins (6 males) developed diabetes, on follow-up, between 1 and 156 mo (median 14 mo) after serum collection. Their mean ± SE age at sampling was 15.2 ± 1.8 yr, and at diagnosis of diabetes, 18.3 ± 2.7 yr. Forty-six of the twins studied (24 men) remained nondiabetic on follow up; 31 were >6 yr discordant from the time of diagnosis of their index twin to the time of serum collection (low risk for diabetes), and only 6 were <6 yr discordant. The mean age of the nondiabetic twins at serum collection was 31.1 ± 2.5 yr, when the mean period of discordance of diabetes was 156 ± 18 mo (range 8–528 mo).

A control group of 28 nondiabetic healthy control subjects (12 men, mean age 21.6 ± 2.4 yr) was selected from the local community. These subjects had normal glucose tolerance, no family history of diabetes, and were taking no drugs.

Patients and/or their parents gave informed consent, and the study was approved by the Ethical Committees at Westminster and King's College Hospital.

Antibody analyses. Antibody analyses were performed by individuals blinded to the clinical status of the patients. Rat tissues were used as sources of antigen for analyses of antibodies to GAD and the 64,000-M_r islet antigen. Whole brains were obtained from adult male and female Wistar rats. Islets were isolated from the pancreases of neonatal Wistar rats by collagenase digestion and Percoll-density gradient centrifugation, according to the procedure of Brundstedt et al. (20).

Antibodies to GAD in sera were measured by determining the enzyme activity immunoprecipitated by sera from a soluble extract of rat brain. The method used was a modification of procedures described by Baekkeskov et al. (8). Brains from adult male Wistar rats were homogenized in a 10-fold volume of 1 mM 2-aminoethylisothiouronium bromide, 0.2 mM pyridoxal phosphate, 1 mM EDTA, 1 mM benzamidine, 25 mM potassium phosphate, pH 7 (homogenization buffer), then centrifuged at 100,000 × *g* for 30 min. The extract was diluted to a GAD activity of 2.5×10^{-9} mol · ml^{-1} · h^{-1}, and 50-μl aliquots of the extract were incubated with 12.5 μl test serum for 5 h at 4°C. Immune complexes were isolated on 25 μl protein A Sepharose and washed four times with 1 ml 10 mM HEPES (pH 7.4), 155 mM NaCl, 10 mM benzamidine, 0.5% Triton X-114, 0.5 mg/ml bovine serum albumin, and once in 1 ml homogenization buffer. Protein A Sepharose pellets were incubated for 16 h at 37°C with 25 μl 5 mM L-glutamic acid and 0.125 μCi [1-^{14}C]-L-glutamic acid in homogenization buffer. $^{14}CO_2$ released during the reaction was absorbed to filter papers soaked with 50 μl 1 M hyamine hydroxide in methanol and measured by scintillation counting. Enzyme activity was calculated relative to that of the same standard antibody-positive control serum used in previous analyses of 64K antibody activities (6,16,21). Sera were regarded as positive if the relative antibody activity was >2SD of the activity in sera from a group of 27 nondiabetic control subjects (mean ± SD 4.2 ± 2.9% of positive control). The interassay coefficient of variation was 15.4%.

Antibodies to 50,000-, 40,000-, and 37,000-M_r tryptic fragments of the protein were measured by immunoprecipitation of [^{35}S]methionine-labeled polypeptides from trypsin-solubilized extracts of neonatal rat islets, as previously described (16). Serum samples were regarded as positive for a specific antibody activity if a band corresponding to the appropriate polypeptide could be de-

TABLE 1
Frequencies of diabetes-associated antibodies in study groups

	n	Antibodies			ICA (>4 JDF U)	IAA
		GAD	50K	37K		
Prediabetic twins	12	8 (67)	9 (75)	8 (67)	9 (75)	3 (25)
Nondiabetic twins						
<6 yr discordant	15	1 (7)	1 (7)	1 (7)	1 (7)	0 (0)
>6 yr discordant	31	5 (16)	6 (19)	0 (0)	4 (13)	2 (6)
Control subjects	28	1 (4)	1 (4)	0 (0)	1 (4)	0 (0)

Values are *n* with percentages in parentheses. GAD, glutamic acid decarboxylase; ICA, islet cell antibody; IAA, insulin autoantibody.

tected on the autoradiogram. Antibody activities were quantified by densitometric scanning of bands on autoradiograms (50,000-M_r band for 50K antibodies and 37,000-M_r band for 37K antibodies), expressing the band density relative to that of the standard 64K antibody–positive control serum included in each experiment.

The presence of ICAs in undiluted sera was assessed by indirect immunofluorescence on 4-μm cryostat sections of blood group O human pancreas, as previously described (2). Positive samples were titrated to end point in doubling dilutions in phosphate-buffered saline. Local standard sera calibrated to 2, 4, 8, 16, 32, and 80 Juvenile Diabetes Foundation (JDF) U were included in each assay. End-point titers of test samples were converted to JDF U by comparison with a standard curve of $\log_2$ JDF U versus $\log_2$ of end-point titer of the standard sera. The threshold of ICA detection was 5 JDF U.

IAAs were measured by a liquid-phase radioligand binding assay as previously described (22). Corrected binding was determined by subtracting the binding after incubation with excess unlabeled insulin from the binding with label alone. Corrected binding levels >3SD of the range obtained with normal sera were regarded as IAA^+. The interassay coefficient of variation was 8.6%.

Glucose tolerance tests. Oral glucose tolerance tests were performed on all nondiabetic twins and control subjects. Glucose (75 g or 1.75 g/kg) dissolved in 300 ml water was consumed over 4 min and blood samples drawn at −10, 0, 30, 60, 90, and 120 min. Whole-blood glucose was analyzed with a glucose oxidase method.

Statistical analysis. The degree of association between antibody activities was tested by linear regression analysis. The significance of differences among frequencies of antibody activities in populations was determined by χ^2 analysis with Yates' correction or by Fisher's exact test, as appropriate. The significance of differences among antibody levels was determined by the Mann-Whitney *U* test. Differences were considered significant at $P < 0.05$.

RESULTS

Antibodies to rat brain GAD, to tryptic fragments of islet 64,000-M_r antigen, ICA, and IAA were analyzed in sera from 58 nondiabetic twins, of whom 12 have subsequently developed diabetes. Table 1 summarizes the results of antibody analyses, and Table 2 shows data for individual subjects. GAD antibodies were detected in 8 of the prediabetic twins (mean ± SE of positives 44.5 ± 17.7% of positive control serum) and 9 were positive for 50K antibodies (50.4 ± 10.8%). A strong positive correlation was observed between GAD antibodies and 50K antibodies in sera from prediabetic twins ($r = 0.88$, $P < 0.001$). Antibodies to 37,000- and 40,000-M_r fragments were detected in 8 of 12 prediabetic twins (mean of positives 77.6 ± 14.5%) and showed no association with GAD antibodies. All 12 prediabetic twins possessed antibodies to at least one of the antigenic fragments. ICAs were detected in 9 prediabetic twins (median titer of positives 30 JDF U, range 5–>80 JDF U), of whom 6 had titers >20 JDF U. ICAs were not associated with GAD antibodies or 50K antibodies in the prediabetic twins but showed a significant correlation with 37K antibodies ($r = 0.64$, $P < 0.05$). Only 3 prediabetic twins were IAA^+.

The frequency of each antibody specificity was significantly lower in the twins who remain nondiabetic ($P < 0.05$). GAD antibodies were detected in 6 twins who were nondiabetic (mean of positives 36.6 ± 11.5%), 5 of whom were >6 yr discordant from the time of diagnosis of their diabetic twin (long-term discordant) and were regarded as having a low risk for disease (Table 1). The 50K antibodies were detected in 7 nondiabetic twins (60.7 ± 15.1%), 6 of whom were long-term discordant. Five of 7 50K antibody–positive nondiabetic twins were also positive for GAD antibodies. Only 1 of 46 nondiabetic twins was positive for 37K antibodies. The frequency of 37K antibodies was significantly lower than that of 50K antibodies in nondiabetic twins ($P < 0.05$) and significantly lower than both GAD antibodies and 50K antibodies in long-term discordant twins ($P < 0.05$). ICAs were detected in 5 nondiabetic twins, including 1 positive for both GAD antibodies and 50K antibodies and the 1 individual positive for 37K antibodies (Table 2). ICAs detected in this group were all low titer (7–9 JDF U), significantly lower than in ICA^+ individuals in the prediabetic group ($P < 0.05$). Two nondiabetic twins were IAA^+. All but 1 of the nondiabetic twins who were positive for one of the antibody specificities are >6 yr discordant

TABLE 2
Individual immunological data for antibody-positive twins

Subject no.	Age at sample (yr)	Sex	Antibodies (% control) GAD	50K	37K	ICA (JDF U)	IAA (% binding)	Period from diabetes (mo)*
Prediabetic twins								
1	14	F	161	116	66	13	–	156
2	28	F	32	48	–	–	–	132
3	18	M	66	88	–	13	–	58
4	20	F	36	35	42	30	–	36
5	13	M	16	35	32	>80	–	19
6	13	M	–	15	–	5	–	14
7	16	F	–	–	152	>80	–	9
8	12	F	–	–	56	30	–	6
9	12	F	17	52	125	>80	0.98	6
10	18	M	–	–	81	–	–	1
11	13	M	13	21	67	>80	0.95	1
12	3	M	15	44	–	–	0.88	1
Nondiabetic twins								
13	11	M	42	62	–	–	–	8
14	21	M	–	–	18	8	–	35
15	21	M	–	–	–	7	–	75
16	19	M	32	96	–	–	–	104
17	39	M	12	19	–	–	–	132
18	20	M	–	–	–	9	–	141
19	25	M	–	75	–	–	–	179
20	29	F	–	23	–	–	–	180
21	30	F	16	27	–	–	–	191
22	27	F	–	–	–	8	–	216
23	63	F	–	–	–	–	0.36	238
24	55	F	28	–	–	–	–	259
25	47	M	90	123	–	9	–	288
26	67	F	–	–	–	–	0.35	348

Immunological data are given for each individual positive for the antibody specificities tested. –, subject was negative for the particular antibody specificity; GAD, glutamic acid decarboxylase; ICA, islet cell antibody; IAA, insulin autoantibody.
*The period from time of serum sample to onset of diabetes for prediabetic twins and period of discordance for nondiabetic twins.

from diagnosis of the index twin. This 1 individual (twin 13 in Table 2) had both GAD and 50K antibodies.

All 12 prediabetic twins had at least one of the five antibody specificities studied; 10 twins had two or more and 5 twins had four or more specificities (Table 2). In contrast, only 13 of 46 twins who remain nondiabetic had at least one of the antibodies; 2 had two of the specificities, none had more than two. There was a significant tendancy for prediabetic twins to have more antibody specificities than for twins who have not developed diabetes ($P < 0.001$).

Impaired tolerance to oral glucose was detected in 6 of 46 nondiabetic twins. Four of these were positive for antibodies to islet cell components; 3 possessed both GAD and 50K antibodies (twins 13, 16, and 21 in Table 2); and the fourth was positive for ICA (twin 15 in Table 2). Glucose tolerance is normal in 4 of these 6 twins, the remaining 2 have antibodies to GAD and the 50,000-M_r fragment (twins 13 and 21).

A control group of 28 nondiabetic subjects with no family history of diabetes were also analyzed for the various antibody specificities. One of this group was positive for both GAD antibodies (49% positive control) and 50K antibodies (45% positive control), and a different subject was ICA$^+$ (7 JDF U). All 28 were negative for 37K antibodies and for IAA, and all had normal glucose tolerance.

DISCUSSION

Previous studies have suggested that the presence of antibodies to a 64,000-M_r islet cell protein represents an early and specific marker for the subsequent development of IDDM (5,7). However, the laborious nature of the 64K antibody assay has restricted its use in studies of antibody frequency in high-risk groups and in the population at large. The tentative identification of the antigen as one or more isoforms of GAD should facilitate the development of simpler assays for antibodies to the enzymes (8). Consistent with the proposal that the 64,000-M_r antigen has GAD activity, we recently demonstrated a strong correlation ($r = 0.92$, $P < 0.001$, by linear regression analysis) between antibodies to the islet 64,000-M_r antigen and brain GAD in sera from newly diagnosed diabetic patients (14). In this article we find a similar strong association between GAD antibodies and 50K antibodies in prediabetic twins. However, nondiabetic twins were identified who were discordant for these antibodies (twins 19, 20, and 24 in Table 2). Antibodies in these subjects may specifically recognize GAD isoforms that display tissue-specific differences in expression or differences in their subcellular localization. In this study, GAD antibodies and 50K antibodies were detected with rat brain cytosol and rat islet membranes, respectively, as sources of antigen. Two isoforms of GAD (GAD_{65} and GAD_{67}) may be recognized by antibodies in diabetic

patients, potentially by different antibodies, and these show differences in membrane association and tissue specificity (9–11). Studies with purified proteins are required to further characterize autoimmune responses to the different isoforms of GAD and to determine the value of isoform-specific antibodies as markers for disease.

ICA, GAD antibodies, and the two distinct antibodies to tryptic fragments of islet 64,000-M_r antigen were all present at similar frequencies in prediabetic twins. Neither GAD antibodies nor 50K antibodies were associated with ICA, and several individuals with high GAD and high 50K antibody activities were ICA$^-$ (Table 2). Thus, GAD is probably not a major antigen recognized by ICA in these individuals. In contrast, a significant correlation was observed between 37K antibodies and ICA. Further studies are required to determine whether 37K antibodies can contribute to the ICA staining on pancreatic sections.

IAAs were detected at a significantly lower prevalence than other antibodies. From this and earlier studies (7,23), it appears that IAA assayed by liquid-phase procedures has a lower sensitivity as a marker for diabetes development than GAD antibodies or ICA. Prediabetic twins had more antibody specificities to islet cell components than twins who did not develop diabetes. The presence of antibodies that recognize several islet cell antigens may be a better marker of diabetes development than antibodies to any one antigen alone.

All antibody activities were present at significantly lower frequencies in twins who remain nondiabetic. Nevertheless, 13 of 46 nondiabetic twins had at least one of the antibody specificities. Four of the nondiabetic twins positive for ICA or GAD antibodies had impaired glucose tolerance, which may be indicative of β-cell dysfunction, but two of these show normal glucose tolerance. All but one of the nondiabetic twins positive for antibodies to islet components are >6 yr discordant for diabetes with their twin and therefore have a low risk for disease development. There is evidence that some genetically susceptible individuals may exhibit an active autoimmune process that may remit or may not achieve sufficient severity for precipitation of disease but in whom immunological or metabolic abnormalities may be transiently apparent (24,25). This may explain, in part, the presence of antibodies to islet cell components in individuals who are unlikely to develop disease.

One control subject was positive for both GAD antibodies and 50K antibodies; a different control subject was ICA$^+$. Both ICA and antibodies to the intact GAD antigen, although rare, can appear in individuals who have very low risk for diabetes development. However, observations in this study suggest that analysis of several serum antibodies can provide relatively accurate markers for disease development. First, all ICA$^+$ nondiabetic subjects had antibodies at low titer, whereas the 6 twins with ICA titers >20 JDF U all developed diabetes. This finding is consistent with the results of an earlier study of ICA in first-degree relatives of diabetic patients (2), in which the predictive value of ICA was improved on increasing the threshold of antibody positivity. This does not appear to be the case for GAD or 50K antibodies because, in contrast to ICA, the antibody levels in antibody-positive prediabetic and nondiabetic twins were similar. Second, only 1 of 9 twins who had 37K antibodies has not developed diabetes. This twin was also ICA$^+$ and was 35 mo discordant for diabetes with his twin at the time of serum collection (high risk for diabetes) but is now >6 yr discordant. He has a consistently impaired first-phase insulin response to intravenous glucose (data not shown), but has maintained normal tolerance to oral glucose. All control individuals tested to date have been negative for 37K antibodies. Because these antibody activities might recognize epitopes that are hidden on the native 64,000-M_r antigen complex, considerable β-cell damage and degradation of antigen may be required to generate an immune response to these epitopes. Alternatively, antibodies to 37,000- and 40,000-M_r tryptic fragments could be directed against a separate protein and be the result of a distinct immune response. In any case, the presence of 37K antibodies may represent a better marker for ongoing β-cell destruction than antibodies to GAD.

The absence of 37K antibodies and high-titer ICA in long-term discordant nondiabetic twins, combined with the high frequencies of these antibodies in prediabetic twins, indicates that these antibodies represent highly specific markers for diabetes development in a population that has genetic susceptibility to IDDM. Further analyses of 37K antibodies in family members of IDDM patients and the general population are important. Studies of schoolchildren indicate that the predictive value of ICA in the general population may be significantly lower than that calculated for twins or siblings of diabetic patients, who may share disease susceptibility genes with their relatives (26–28). A combination of several genetic and antibody markers may be required to predict diabetes in the general population. Further studies in genetically susceptible groups, such as siblings of diabetic patients, are required to determine whether 37K antibodies are accurate markers of diabetes development in other population groups or whether other markers, or combinations of markers, represent the best immunological markers for disease development.

In conclusion, antibody markers such as ICA, IAA, and antibodies to 64,000-M_r antigen can be predictive of diabetes in genetically susceptible populations but may also identify false positive individuals who do not progress to disease. This problem may be overcome by further dissecting the islet antibody–antigen system and determining whether new antibody specificities can act as better disease markers. Analysis not only of antibodies to specific antigens but also to specific determinants on antigens may be required if the best predictive markers for diabetes development are to be identified. The molecular cloning of the components of the 64,000-M_r antigen should facilitate studies to identify the epitopes recognized by the distinct diabetes-associated antibodies and will enable the development of epitope-specific, as well as antigen-specific, antibody-screening assays.

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