High Titer of Autoantibodies to GAD Identifies a Specific Phenotype of Adult-Onset Autoimmune Diabetes

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OBJECTIVE — The aim of the present study was to define heterogeneity of adult-onset autoimmune diabetes based on characterization of GAD antibodies (GADAs).

RESEARCH DESIGN AND METHODS — Patients enrolled in a nationwide survey, the Non Insulin Requiring Autoimmune Diabetes (NIRAD) Study, have been screened for GADAs and IA-2 antibodies (IA-2As) and further characterized for GADA titer, antibodies to thyroid peroxidase (TPO), and HLA DRB1-DQB1 polymorphisms.

RESULTS — Of 4,250 consecutive type 2 diabetic patients, 4.5% had either GADAs and/or IA-2As. Patients with autoimmune diabetes showed a clinical phenotype significantly different from that of type 2 diabetes, including higher fasting glucose and A1C, lower BMI and uric acid, lower prevalence of metabolic syndrome and its components, and higher frequency of TPO antibodies. More interestingly, analysis of GADA titers showed a bimodal distribution that identified two subgroups of patients with high (>32 GADA arbitrary units) and low (≤32 GADA arbitrary units) GADA titers. Compared with those with low GADA titers, patients with high GADA titers had more prominent traits of insulin deficiency and a profile of more severe autoimmunity resulting in higher A1C, lower BMI, a lower prevalence of metabolic syndrome and its components ($P < 0.02$ for all), a higher prevalence of IA-2As, TPO antibodies ($P < 0.003$ for both), and DRB1*03-DQB1*0201 (50 vs. 26.8%, $P = 0.001$), and a decreasing frequency of DQB1*0602 and DRB1*0403 (from type 2 to low and to high GADA titer autoimmune diabetes; $P < 0.001$ for trend for both comparisons).

CONCLUSIONS — GADA titers identify two subgroups of patients with adult-onset autoimmune diabetes having distinct clinical, autoimmune, and genetic features.

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Abbreviations: GADA, GAD antibody. IA-2A, IA-2 antibody; ICA, islet cell antibody; NIRAD, Non Insulin Requiring Autoimmune Diabetes; TPO, thyroid peroxidase; U, arbitrary units.

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

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Slowly progressive autoimmune diabetes in adults often presents with a clinical phenotype indistinguishable from that of classic type 2 diabetes. This form of diabetes, variably referred to as latent autoimmune diabetes in adults (1,2), slow progressing insulin-dependent diabetes (3), non–insulin-requiring autoimmune diabetes (4), or type 1.5 diabetes (5), is usually diagnosed by presence of islet autoantibodies, namely antibodies to GAD (GADAs), with or without associated cytoplasmic islet cell antibodies (ICAs) and/or protein tyrosine phosphatase IA-2 antibodies (IA-2As) (6,7).

The prevalence of islet autoantibodies in patients with clinically diagnosed type 2 diabetes was investigated in several studies, with reports of 4–10% having markers of islet autoimmunity (6–10). Patients identified by these autoantibodies show a clinical phenotype intermediate between type 1 and type 2 diabetes (8) and a higher risk of progression to insulin-requiring diabetes compared with patients without antibodies (6,7). Nonetheless, several aspects of adult-onset autoimmune diabetes remain to be clarified. A point of major importance is its heterogeneity, clearly emerging from the spectrum of clinical phenotypes, the possible association with other endocrine autoimmunities, the composite autoantibody profile, and the underlying polymorphic genetic background. Molecular characterization of islet autoantibodies, including determination of titer, proved to be a valuable tool for the understanding of the natural history of the autoimmune process and the prediction of future insulin dependence in juvenile-onset type 1 diabetes (11). GADAs are the single most sensitive antigen-defined autoantibody markers of autoimmune diabetes (12), and their titration was used to stratify the risk of progression to insulin dependence in adult-onset diabetes (7). The aim of the present study was to determine whether GADA titers may define heterogeneity within adult-onset autoimmune diabetes, with a specific focus on clinical phenotype, islet autoantibody response, associ-

ation with thyroid autoimmunity, and underlying HLA class II polymorphisms.

RESEARCH DESIGN AND METHODS

— The Non Insulin Requiring Autoimmune Diabetes (NIRAD) Study is a nationwide survey sponsored by the Società Italiana di Diabetologia with the aim of assessing the prevalence and characteristics of autoimmune diabetes within adult patients attending diabetes clinics in Italy with a clinical diagnosis of non–insulin-requiring diabetes. Between February 2001 and June 2004, 4,250 type 2 diabetic patients were recruited from 83 diabetes centers equally distributed in the entire mainland and island Italian territory. Inclusion criteria were *1*) diagnosis of diabetes according to the American Diabetes Association (13), no insulin requirement, and no evidence of ketosis from diagnosis to screening time and *2*) disease duration between 6 months and 5 years. Exclusion criteria included prior insulin therapy, pregnancy, and the presence of any other severe disease. The study was approved by all local ethics committees, and written informed consent was obtained from all patients.

Clinical and biochemical measurements

In all patients, BMI, blood pressure, and waist circumference were measured; blood samples were obtained for local biochemical and centralized autoantibody measurements and genetic typing. Biochemical measures included fasting glucose, total and HDL cholesterol, triglycerides, uric acid, and A1C; all of these assays were performed with local routine quality-controlled programs using international reference standards. Patients were classified with regard to the metabolic syndrome according to the Adult Treatment Panel III criteria (14).

Autoantibody measurements

GADAs and IA-2As were measured centrally by reference laboratories in Milan and Rome, respectively, using a radiobinding assay with in vitro–translated [^{35}S]methionine-labeled GAD_{65} (15) and $IA\text{-}2_{IC}$ (amino acids 605–979) (16). Results for GADA were converted into arbitrary units (U) by extrapolation from a standard curve with a local standard designated 100 U. Results for IA-2As were expressed as an index defined as follows: (sample counts per minute – negative standard control counts per minute)/(positive standard control counts per minute – negative standard control counts per minute). The thresholds for positivity were determined from the 99th centile of control subjects and corresponded to 3 U for GADAs and index 0.010 for IA-2As. The following results were obtained for these GADA and IA-2A assays at the first, second, and third assay proficiency evaluations of the Diabetes Antibody Standardization Program (17) performed between 2002 and 2005: GADA sensitivity 84, 86, and 88%; GADA specificity 97, 97, and 92%; IA-2A sensitivity 60, 62, and 70%; and IA-2A specificity 100, 99, and 99%, respectively. The intra- and interassay coefficients of variation of GADA for control samples designated at 10 GADA U were 9 and 17%, respectively, and for $IA\text{-}2_{IC}$ were 4.8 and 9.9%, respectively. Specificity of antibody binding to GAD was tested in a subset of patients with intermediate and low GADA titers by an inhibition assay using an excess of unlabeled GAD_{65} at a final concentration of 60 μg/ml. Human recombinant GAD_{65} was produced in our laboratory by Ezio Bonifacio by baculovirus, purified on a DEAE-Sepharose column, and dialyzed before testing (18). Binding to GAD was considered as specific in the presence of either complete inhibition with residual binding ≤0.3 U or Δ binding (total – inhibited) ≥3 U. Thyroid peroxidase (TPO) antibodies were measured centrally in Milan, Italy, by a radioimmunoassay using a commercial kit (Medipan, Berlin, Germany).

HLA genotyping

Genomic DNA was extracted using the salting-out method. HLA-DRB1 and -DQB1 typing was performed by PCR; high-resolution typing for DRB1*04 and DQB1 loci was performed using allele group–specific amplifications. A reverse line blot method, kindly provided by H.A. Erlich and T. Bugawan (Roche Molecular System, Alameda, CA), was used as the detection system (19). HLA genotypes were classified in three risk categories (high, moderate, and low), based on the absolute risk values for type 1 diabetes previously estimated in the Italian population (20).

Statistical analysis

Statistical analysis was performed using SPSS statistical software (version 13; SPSS, Chicago, IL). Data are expressed as frequencies or as means ± SD. Frequency differences were compared using the χ^2 test (with Yates' continuity correction) or Fisher's exact test when appropriate. Statistical differences between groups for quantitative variables were investigated using multiple linear regressions. Comparisons were adjusted for age of recruitment, duration of disease, sex, and therapy. Patients with GADAs were divided into two groups representing the titer modes, and two dummy variables were created to include the groups (high GADA and low GADA titers, with type 2 diabetes being the reference category) of patients among the predictors in the multiple regression analysis. Data for triglycerides and HDL were transformed using log base 10 to normalize their distributions. The interaction between GADA titers and sex for the presence of TPO antibodies was evaluated by logistic regression analysis. The HLA DRB1-DQB1 allele frequencies were in Hardy-Weinberg equilibrium.

RESULTS

Autoantibody prevalence and clinical association

Of the 4,250 patients recruited, 193 (4.5%) had either GADAs or IA-2As. Of these, 191 (4.4%) had GADAs, 39 (0.91%) had IA-2As, and 37 (0.87%) had both; IA-2As in the absence of GADAs were found in 2 patients only. Patients with GADAs and/or IA-2As are hereafter defined as having autoimmune diabetes; patients with no antibodies are hereafter defined to as having "classic" type 2 diabetes. Patients with GADAs ($n = 191$) and a twofold number of age- and sex-matched patients with type 2 diabetes ($n = 382$) were further characterized by HLA DRB1-DQB1 genotypes and TPO antibody measurement. Compared with patients with type 2 diabetes, patients with autoimmune diabetes showed similar sex distribution but significantly higher fasting glucose and A1C, significantly lower age at diagnosis, BMI and waist circumference, total cholesterol, uric acid, and triglycerides, and higher HDL cholesterol levels (Table 1); a lower prevalence of the metabolic syndrome (58.6% in autoimmune vs. 67.6% in type 2 diabetes, $P = 0.01$); a higher prevalence of TPO antibodies (27% in autoimmune vs. 10.5% in type 2 diabetes, $P < 0.001$); different HLA class II distribution, with a higher frequency of moderate- and high-risk genotypes (22.5 and 8.4% in autoimmune vs. 11.2 and 1.2% in type 2 diabetes, respectively; all $P < 0.001$); and a higher frequency of DRB1*04-DQB1*0302 and

Table 1—*Clinical characteristics of patients with autoimmune and type 2 diabetes*

	Autoimmune diabetes	Type 2 diabetes	*P**
n (male/female)	100/93	2,110/1,947	
Age of recruitment (years)	52.6 ± 13	57.8 ± 10.88	<0.001
Age of diagnosis (years)	50.3 ± 12.83	55.64 ± 10.81	<0.001
A1C (%)	7.5 ± 1.7	6.8 ± 1.6	<0.001
BMI (kg/m^2)	27 ± 5.16	29.9 ± 5.4	<0.001
Waist circumference (cm)	94.6 ± 12.81	101 ± 13.29	<0.001
Fasting glucose (mg/dl)	168.1 ± 58.1	149.38 ± 44.47	<0.001
Triglycerides (mg/dl)	144 ± 104	161 ± 119	0.02
HDL cholesterol (mg/dl)	50 ± 13	47.7 ± 12.5	0.01
Total cholesterol (mg/dl)	197 ± 46.1	209 ± 43.3	0.02
Uric acid (mg/dl)	4.47 ± 1.43	5.13 ± 1.44	<0.001

Data are means ± SD unless otherwise indicated. Triglycerides were also corrected for A1C. *All comparisons are adjusted for age of recruitment, duration of disease, sex, and therapy.

DRB1*03-DQB1*0201 haplotypes (24 and 38.2% in autoimmune diabetes vs. 10 and 16.5% in type 2 diabetes, respectively; all $P < 0.001$).

GADA titers and clinical association

The distribution of GADA titers in patients with autoimmune diabetes was independent of diabetes duration and showed a bimodal distribution. Consistent with this observation, patients with autoimmune diabetes were divided into subgroups representing the two distributions, namely low (taken to be ≤32 U) and high (>32 U) GADA titers (Fig. 1). An inhibition assay was performed in 94 of 97 samples with low GADA titers; of these, antibody binding for GAD was specific in 88 and nonspecific in 6. Compared with those with low GADA titers, patients with high GADA titers had more prominent traits of insulin deficiency and a profile of more severe autoimmunity resulting in significantly higher A1C and significantly lower BMI, total cholesterol, triglycerides, and prevalence of the metabolic syndrome (Table 2). Moreover, patients with high GADA titers showed a significantly higher prevalence of IA-2As (Table 3). TPO antibody prevalence showed a different behavior depending on sex: in men, TPO antibody levels were significantly more frequent in patients with high GADA titers than in those with both type 2 diabetes and low GADA titers, who showed comparable frequencies; conversely, in women with low GADA titers, TPO antibody levels were intermediate between those in patients with high GADA titers and those with type 2 diabetes. Higher levels of TPO antibody titers were observed in patients with high GADA titers than in those with low GADA titers and type 2 diabetes ($P < 0.001$ for linear trend) (Table 3). Compared with patients with type 2 diabetes, differences in age of diagnosis, BMI, waist circumference, fasting glucose, A1C, and uric acid were more pronounced in patients with high GADA titers than with low GADA titers ($P < 0.001$ for linear trend). On the other hand, in patients with low GADA titers, total cholesterol, triglycerides, and the frequency of the metabolic syndrome were similar to those in patients with type 2 diabetes (Table 2).

GADA titers and HLA class II

An increasing linear trend in the prevalence of high/moderate HLA risk genotypes was observed for patients with type 2 diabetes compared with patients with low GADA titers and high GADA titers ($P < 0.001$ for both comparisons). Patients with high GADA titers displayed the highest frequency of DRB1*03-DQB1*0201 (50%) compared with those with low GADA titers (26.8%, $P = 0.001$) and type 2 diabetes (16.5%, $P < 0.001$).

In contrast, the DRB1*04-DQB1*0302 frequency was similarly elevated in patients with either high or low GADA titers compared with patients with type 2 diabetes ($P < 0.001$ for both comparisons). DRB1*04 subtyping showed a linear trend for DRB1*0403,

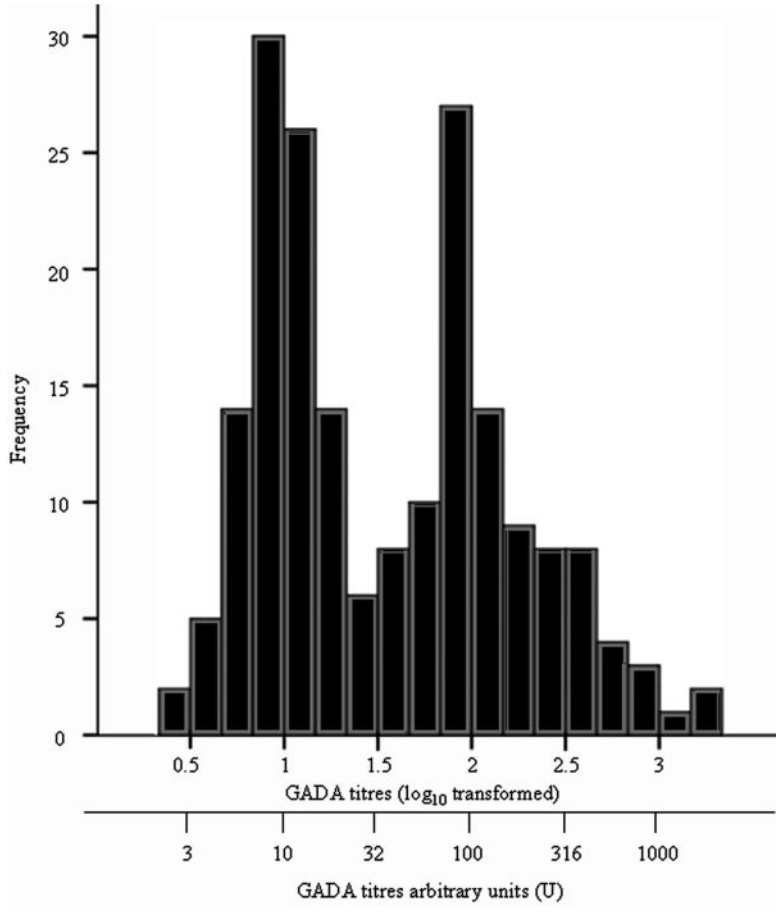

Figure 1—*Bimodal distribution of GADA titers (log_{10} transformed) in patients with autoimmune diabetes.*

Table 2—*Clinical characteristics of patients with autoimmune diabetes with high and low GADA titer and type 2 diabetes*

	High GADA titer autoimmune patients	Low GADA titer autoimmune patients	Type 2 diabetic patients	*P* value* High GADA vs. type 2	*P* value* Low GADA vs. type 2	*P* value* High vs. low GADA	*P* value for trend
n (male/female)	49/45	50/47	2,110/1,947				
Age of diagnosis (years)	49.1 ± 12.29	51.5 ± 13.13	55.6 ± 10.81	<0.001	0.002		<0.001
BMI (kg/m^2)	26.29 ± 5.16	28.43 ± 5.01	29.9 ± 5.4	<0.001	0.03	0.002	<0.001
Waist circumference (cm)	92.86 ± 12.6	96.37 ± 13.37	101 ± 13.29	<0.001	0.01		<0.001
Fasting glucose (mg/dl)	170.4 ± 63.4	166 ± 53	149.38 ± 44.47	<0.001	0.001		<0.001
A1C (%)	7.8 ± 1.7	7.2 ± 1.8	6.8 ± 1.6	<0.001	0.01	0.02	<0.001
Total cholesterol (mg/dl)	186 ± 44.8	207 ± 47	209 ± 43.3	<0.001		0.01	
HDL (mg/dl)	49.7 ± 14.4	50.5 ± 12.3	47.7 ± 12.5				
Triglycerides (mg/dl)	116 ± 106	171 ± 102	161 ± 119	<0.001		0.005	
Uric acid (mg/dl)	4.38 ± 1.71	4.62 ± 1.16	5.13 ± 1.44	<0.001	0.002		<0.001
With metabolic syndrome	43 (45.7)	69 (71.1)	2742 (67.6)	<0.001		0.01	

Data are means ± SD or *n* (%) unless otherwise indicated. Triglycerides were also corrected for A1C. *All comparisons are adjusted for age of recruitment, duration of disease, sex, and therapy.

with the highest frequency in patients with type 2 diabetes and decreasing in those with low GADA titer and then in those with high GADA titer autoimmune diabetes, which showed the lowest frequency, with a pattern similar to that observed for DQB1*0602 ($P < 0.001$ for both).

CONCLUSIONS — Data reported in the last few decades have supported the concept that autoimmune diabetes, although generally considered a childhood or juvenile-onset disease, is indeed an "all age" disease, with diagnosis in >40% of patients at >30 years of age (21). However, whereas for those with early-onset disease, the term "type 1 diabetes" is universally applied, several other terms, including latent autoimmune diabetes in adults (1,2), non–insulin-requiring diabetes (4), and type 1.5 diabetes (5), have been proposed to define adult-onset autoimmune diabetes.

The reason for this lack of uniformity in definition is mainly related to the heterogeneity of the clinical presentation, ranging across the whole spectrum between classical phenotypes of type 1 and type 2 diabetes. The findings of our study provide novel insights into this matter: the bimodal distribution of GADA titers identifies a first group of patients with a

Table 3—*TPO antibodies, IA-2As, and HLA class II in patients with autoimmune diabetes with high and low GADA titer and type 2 diabetes*

	High GADA titer autoimmune diabetes	Low GADA titer autoimmune diabetes	Type 2 diabetes	*P* value* High GADA vs. type 2	*P* value* Low GADA vs. type 2	*P* value* High vs. low GADA	*P* for trend
n (male/female)	49/45	50/47	198/184				
TPO antibodies							
Male	17 (34.7)	2 (4)	11 (5.6)	<0.001		<0.001	
Female	18 (40)	14 (29.8)	29 (15.8)	<0.001	0.04		<0.001
TPO antibody titers (units/ml)	836 ± 1521	653 ± 1388	213 ± 1018	<0.001	<0.001		<0.001
IA-2 antibodies	13/11 (25.5)	4/4 (8.2)				0.002	
High-risk HLA genotypes†	8 (8.5)	8 (8.3)	4 (1.1)	<0.001	<0.001		<0.001
Moderate-risk HLA genotypes‡	23 (24.5)	20 (20.6)	43 (11.2)	0.002	0.02		<0.001
Low-risk HLA genotypes§	63 (67)	69 (71.1)	335 (87.7)	<0.001	<0.001		<0.001
DRB1*04-DQB1*0302 positive	22 (23.4)	24 (24.7)	38 (10)	<0.001	<0.001		
DRB1*0401-DQB1*0302	5 (22.7)	7 (29.2)	8 (21)				
DRB1*0402-DQB1*0302	7 (31.8)	5 (20.8)	7 (18.4)				
DRB1*0403-DQB1*0302	1 (4.5)	5 (20.1)	13 (34.2)	0.01			<0.001
DRB1*0404-DQB1*0302	4 (18.2)	4 (16.7)	6 (15.8)				
DRB1*0405-DQB1*0302	6 (27.3)	5 (20.8)	7 (18.4)				
DRB1*03-DQB1*0201 positive	47 (50)	26 (26.8)	63 (16.5)	<0.001	0.02	0.001	<0.001
DQB1*0602	4 (4.3)	8 (8.2)	50 (13)	0.01			<0.001

Data are *n* (%) or means ± SD unless otherwise indicated. *$P = 0.01$ interaction between GADA titers and sex on the presence of TPO antibodies. †High: DRB1*03-DQB1*0201/DRB1*04-DQB1*0302 genotype (DRB1*04 different from 0403, 06, 11). ‡Moderate: DRB1*04-DQB1*0302/DRB1*04-DQB1*0302, DRB1*03-DQB1*0201/DRB1*03-DQB1*0201, DRB1*04-DQB1*0302/X, and DRB1*03/X (X different from DRB1*03, DRB1*04-DQB1*0302 [DRB1*04 not 0403, 06, 11], or DQB1*0602/03) genotypes. §Low: other genotypes.

high GADA titer in which the autoimmune process is presumably strong enough to induce diabetes with no major contribution by other concomitant factors and a second group with low GADA titers, reflecting a less intense autoimmune process, with associated features of insulin resistance.

Overall, in our study the prevalence of GADAs and/or IA-2As was 4.5%. Compared with other large studies, this prevalence is less than those reported in some northern European populations such as in the U.K. Prospective Diabetes Study (6,7) and the Botnia Study (8), slightly lower than that of another Italian study (10), and very close to that of the international A Diabetes Outcome Progression Trial (9).

As expected from previous studies, patients with autoimmune diabetes showed a clinical phenotype significantly different from that of classic type 2 diabetes, with the additional evidence of a lower uric acid level in autoimmune patients. Another finding, expected but never investigated in large studies, is the higher frequency of associated thyroid autoimmunity in patients with autoimmune diabetes compared with classic type 2 diabetes. The association of adult autoimmune diabetes with other autoimmune organ-specific disorders, primarily involving the thyroid gland, has been known for decades (22); however, the proportion of patients with such polyendocrine disorders within the whole population of adult autoimmune diabetes has been reported only in one study of limited sample size (23). Our study showed that >1 in 4 patients with adult-onset autoimmune diabetes have markers of associated thyroid autoimmunity compared with 1 in 10 observed in classic type 2 diabetes.

The identification of a bimodal distribution of GADA titers prompted us to test whether this quantitatively measurable marker could be a distinctive trait of heterogeneity within autoimmune diabetes. Autoimmune diabetic patients were then classified into two groups, almost equal in number, having high or low GADA titers and compared with each other and with those with type 2 diabetes. Patients with high GADA titers had more prominent characteristics of insulin deficiency and a profile of more severe and extended autoimmunity as indicated by a higher proportion of associated IA-2As and a higher prevalence and titer of TPO antibodies.

On average, patients with low GADA titers showed intermediate values between patients with high GADA titers and those with type 2 diabetes, with significant linear trends across the three groups for several of the variables measured. In view of the low titer in half of these patients and the frequent finding of GADAs in nondiabetic individuals also (18), the possibility that these autoantibodies were nonspecific was tested by an inhibition assay with an excess of unlabeled antigen in all samples available for retesting. Results showed that in most cases antibody binding was specific for GAD, indicating that autoimmunity in these patients is real, although of low intensity. The features of the mild insulin resistance phenotype displayed by these patients suggest that a facilitating background is needed for the development of diabetes in association with a low-grade autoimmune response.

The heterogeneity based on the GADA titer is supported by the genetic analysis. DRB1*03-DQB1*0201 was found with the highest frequency in patients with high GADA titers, with a decreasing trend in patients with low GADA titers, and then in patients with type 2 diabetes. These findings are consistent with the notion that DRB1*03-DQB1*0201 is also associated with a number of other autoimmune disorders (24,25), being a haplotype conferring susceptibility to autoimmunity as a whole. The occurrence of DRB1*04-DQB1*0302 was significantly increased in autoimmune compared with type 2 diabetes regardless of GADA titer, as previously demonstrated (26), but DRB1*04 subtyping revealed a decreasing linear trend for the frequency of the protective DRB1*0403 allele (27), from type 2 to autoimmune diabetes with low and then high GADA titers, parallel to that observed for the protective DQB1*0602 allele. These novel observations provide additional support to the concept of heterogeneity within autoimmune diabetes in adults.

The pathogenesis of adult-onset autoimmune diabetes has been debated recently (28) with emphasis on the possible role of autoimmunity and insulin resistance as coexistent pathogenetic determinants (29) as previously suggested for the juvenile-onset form of type 1 diabetes (30). Our findings are consistent with this hypothesis, indicating that both autoimmunity and insulin resistance may contribute to the pathogenesis of diabetes in adults with a variable degree of synergism, as reflected by titers of GADA.

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APPENDIX

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