ORIGINAL ARTICLE

Immunogenetic, Clinical, and Demographic Characterization of Childhood Type I Diabetes in New Zealand

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OBJECTIVE — To examine the relationship between genetic susceptibility alleles and islet cell antibodies (ICAs) in type I diabetes.

RESEARCH DESIGN AND METHODS — The human leukocyte antigen (HLA)-DQB1 alleles and ICA levels of all incident type I diabetic cases in patients <20 years of age diagnosed in 1990 and 1991 in Canterbury, New Zealand, were determined.

RESULTS — The mean annual incidence rate for type I diabetes over the 24 months was 19.0/100,000 patient-years (95% confidence interval [CI] 13.5–26.0/100,000), which was considerably higher than rates observed between 1982 and 1989 (11.7/100,000; 95% CI 9.6–14.3/100,000). ICAs ≥10 Juvenile Diabetes Foundation units (JDF U) were present in 84.6% of the subjects, but there was a higher prevalence of ICA-negative (ICA^-) subjects among those diagnosed during the winter months. The frequencies of the susceptibility allele DQB1*0302 and susceptibility genotype 0302/0201 were 71.8% and 43.5%, respectively. Subjects with 0302 tended to be younger (mean age 8.3 ± 5.1 years) than those with nonsusceptibility types (mean age 11.8 ± 4.7 years, $P = 0.056$). The probability of being ICA positive (ICA^+) was not significantly different between subjects with allele 0302 (85.7%) and those without it (81.8%). All seven patients negative for ICA were homozygous for DQB1 nonaspartate-57. There was no clustering of the immunogenetic markers with demographic and clinical characteristics apart from age at diagnosis.

CONCLUSIONS — No direct relationship was observed between DQB1-defined genetic susceptibility and ICA at diagnosis, suggesting that variations at the DQB1 locus are not linked to the expression of this autoimmune marker of β-cell destruction.

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CI, confidence interval; GAD, glutamic acid decarboxylase; HLA, human leukocyte antigen; ICA, islet cell antibody; JDF U, Juvenile Diabetes Foundation units; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Susceptibility or resistance to type I diabetes is associated with human leukocyte antigen (HLA) class II genes, the HLA-D region on chromosome 6 (1,2). The DQ region has been implicated as being most strongly related to type I diabetes susceptibility (3), in particular, polymorphism at the DQB1 locus (4). The presence of an aspartic acid residue at position 57 is associated with protection against type I diabetes in Caucasian populations, because the alleles encoding nonaspartic acid residues are disproportionately prevalent in type I diabetes (5–7). It has been shown that particular alleles and haplotypes account for the observed DQB1 susceptibility (8,9). Allele DQB1*0302 (World Health Organization nomenclature [10], referred to hereafter as 0302, etc.) is the strongest susceptibility allele known for type I diabetes (11,12). The DQB1 genotype 0302/0201 is even more positively associated with type I diabetes. Strong associations have also been established for HLA-DQA1 alleles coding for arginine at position 52 (13–15).

Various studies have explored relationships between ICAs and HLA-DR typing and, more recently, HLA-DQB1 codon 57 in nondiabetic subjects (16,17) and HLA-DR and levels or persistence of ICA in type I diabetes (18–20). No association has been found between DR types and ICAs in type I diabetes. However, a correlation between DR/DQ types and anti-glutamic acid decarboxylase (GAD) antibodies has been reported (21), low-risk DQ antigens being associated with reduced prevalence of anti-GAD antibodies. In the population studied in Canterbury, New Zealand, there has been significant temporal variation in incidence of childhood diabetes (22), with a marked increase in attack rates between 1990 and 1993. One possible explanation for this variation is the introduction or removal of triggering events from the environment. These environmental factors could express themselves on different cohorts of the population from one year to

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the next, depending on the absence or presence of specific susceptibility immunogenetic characteristics (23). Immunogenetic characterization may thus identify subsets of cases presenting with different etiologies, distinguishable by their association with particular combinations of disease markers. This led us to propose that type I diabetes cases over this time of high incidence could be disproportionately represented by subjects not showing ICA or genetic susceptibility characteristics. The aim of this study, therefore, was to determine the frequencies of and examine relationships between ICA and DQB1 haplotypes in demographically and clinically characterized type I diabetes incident cases in the Canterbury population during this period of increased attack rate.

RESEARCH DESIGN AND METHODS

Subjects

All new type I diabetes cases in patients >20 years of age diagnosed in 1990 and 1991 in Canterbury, New Zealand, were prospectively studied. Ethical approval was granted by the Ethics Committee of this institution, and consent for participation in the study was obtained for all subjects at diagnosis. Ascertainment of new diagnoses of type I diabetes in this geographical area is 100% for this age group. All pediatric cases are hospitalized for initial inpatient care, and cases in the 13- to 19-year-old age-group come under the care of the adolescent and adult specialist service. Methods for primary and secondary ascertainment have been established as part of the requirements for research collaborators in the World Health Organization Multinational Diamond Study of Childhood Diabetes (24).

Demographic and clinical parameters

At diagnosis, a patient data sheet was completed by the attending clinician and the patient and/or patient's parents. This provided age, sex, date of diagnosis, glucose and ketone measurements at diagnosis, duration of symptoms, and family history information. Duration of symptoms and family history data was by patient and parent recall.

ICA test

Blood samples were drawn as near as possible to diagnosis for both measurement of ICA and genetic typing of the HLA-DQB1 locus. ICAs were measured by an indirect immunofluorescence assay using blood group O human pancreas. Tissue sections (5 μm) were mounted on aminoalkylsilane coated slides for improved adhesion and were air dried for at least 1 h. The assay incorporated a tissue preincubation step with ICA^- normal serum that improved background staining without interfering in the assay, making assessment of ICA fluorescence easier. Serum samples were diluted in phosphate-buffered saline with aprotinin (Bayer) to inhibit proteolytic activity in the tissue during the incubation period (4). After overnight incubation, the sections were incubated with fluorescein-labeled anti-human IgG (Atlantic Antibodies). Coverslips were mounted with a Dabco mountant to reduce stain fading.

Results were reported in Juvenile Diabetes Foundation units (JDF U), in doubling dilution increments, by reference to an assay standard curve. The assay has a detection limit of 2.5 JDF U, and 10 JDF U is considered the level of significance for positivity. ICA levels were negative (<2.5 JDF U) in 64 healthy children aged ≤20 years. The laboratory participates in the International ICA Proficiency Programme (88% sensitivity and 100% specificity).

HLA-DQB1 typing

Allelic typing of the HLA-DQB1 locus was performed by a polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) technique. The method was based on that developed in Pittsburgh, PA (25), with some modifications.

Genomic DNA was extracted by a rapid preparation method (26) modified for EDTA whole blood using NH_4Cl precipitation and lysis of white cells. The primers for the first PCR (40 μmol/l each) spanned codons 6–13 and 79–86 of the DQB1 sequence, and for the second PCR on the amplification product, the primers spanned codons 14–20 and 79–86 (100 μmol/l each). A "hotstart" PCR technique (27) was used, as well as the two-step procedure, to increase the specificity of amplification. Both of the PCR steps were 40 cycles, with 1 min at each temperature in the denaturation (94°C), annealing (62°C), and extension phases (72°C). Also, extra precautions such as DNase treatment of the PCR premix, use of aerosol-resistant pipette tips, site separation for carrying out the different PCR procedures, and repeat controls and test samples for reproducibility checks were included to eliminate contamination problems.

Allelic assignments were made by digestion of the final PCR product with restriction endonucleases. *Acy* I, *Cfo* I, *Hpa* II (Boehringer Mannheim), and *Tha* I/*Bst*UI (Bethesda Research Laboratories/New England Biolabs) enzymes were used. In addition, *Sau* 961 (Boehringer Mannheim) was used to check assignments of the allele 0302. The digest products were run on Nusieve (FMC Bioproducts) agarose electrophoresis gels and stained with ethidium bromide. The fragment patterns were photographed with a Polaroid camera. Fourteen of the 17 known DQB1 alleles can be typed by this method, since three pairs of alleles cannot be distinguished (viz. 0502 and 0504, 0602 and 0603, and 0604 and 0605).

Statistical analysis

The statistical software package SYSTAT was used. Differences in HLA-DQB1 frequency and in the proportions of subjects positive for ICAs were tested by Fisher's Exact Test. For continuous variables (e.g., age, duration of symptoms, glucose levels), unpaired Student's *t* tests were used. Two-tailed *P* values were used for deter-

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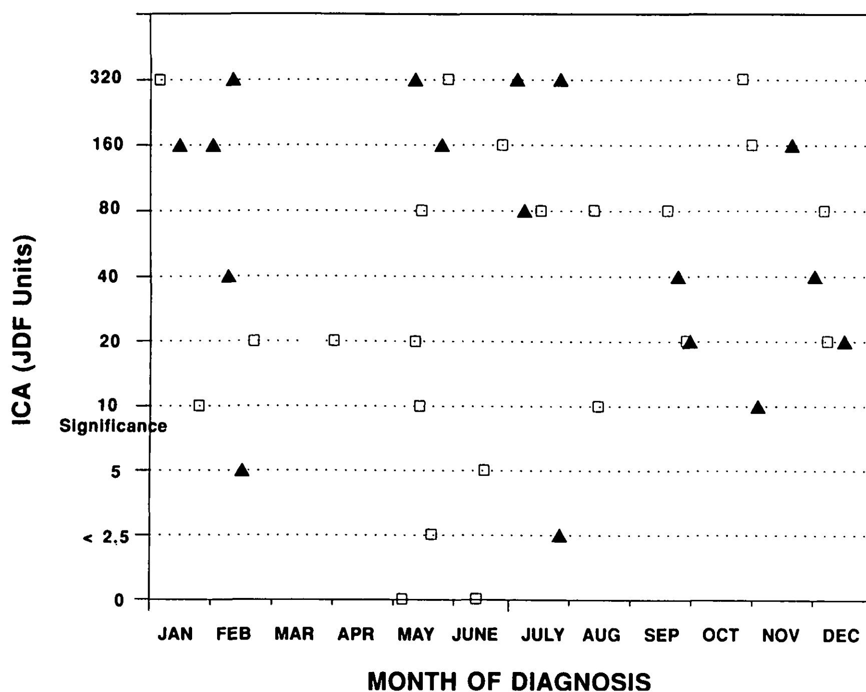

Figure 1—*ICA level and month of diagnosis of type I diabetes cases. Spring: Sept.-Nov.; summer: Dec.-Feb.; autumn: Mar.-May; winter: Jun.-Aug.* □, *male subjects;* ▲, *female subjects.*

mining statistical significance. Data, where appropriate, are expressed as means ± SD. The 95% confidence intervals (CIs) for diabetes incidence rates were calculated assuming a Poisson distribution.

RESULTS

Demographic and clinical data

There were a total of 40 new cases of type I diabetes in the 0- to 19-year-old age-group: 1990 cases $n = 22$, 1991 cases $n = 18$. One woman presenting in 1990, aged 19.7 years, had cystic fibrosis and was excluded from the study because diabetes may have been secondary. The annual incidence rate for these 2 years was 19.0/100,000 patient-years (95% CI 13.5–26.0/100,000), which contrasts with the rate for the period 1982–1989 (11.7/100,000; 95% CI 9.6–14.3/100,000). All subjects were Caucasoid, with three exceptions: two part-Maori sisters and one part-Melanesian girl.

The mean age of the 39 subjects at presentation was 9.2 ± 5.2 years (range 1.2–18.4 years) with 51.3% being <10 years of age. There were 22 male and 17 female subjects. Only four subjects (10.3%) had a first-degree relative with type I diabetes at the time of diagnosis, and there were no first-degree relatives with type II diabetes. The mother of two subjects (sisters who both presented in 1990) had gestational diabetes. Eighteen subjects (46.2%) recalled having no first- or second-degree relatives with diabetes. These 18 subjects were significantly younger (6.8 ± 4.4 years) than those with a family history of diabetes (11.4 ± 4.9 years, $P < 0.01$).

Nineteen subjects (48.7%) reported duration of symptoms of <2 weeks. The parents of one subject judged the symptoms to have been present for ~1 year; otherwise, all reports ranged from 2 days to 5 months. Duration of symptoms was not recorded in two of the older subjects. There was no association of demographic or clinical features with duration of symptoms.

The mean blood glucose level at diagnosis was 28.0 mmol/l (range 14.0–61.0 , SD 11.0). A positive ketone result was recorded in 31 of the 36 subjects for which a measurement was made. The five ketone-negative subjects had ICA levels that were only moderately elevated to 20 JDF U.

Immunogenetic data

A positive ICA result (≥10 JDF U) was obtained in 33 subjects (84.6%). The blood samples for ICA testing were retrieved within 1 month of diagnosis for 37 subjects, most within 1 week of diagnosis. Of the two subjects for which there was a longer delay, one subject measured 5 JDF U 3.5 months after diagnosis and the other was 320 JDF U after 6.5 months.

There was no significant difference between the sexes in ICA levels. There was no correlation between age and ICA level. Figure 1 shows the ICA level and month of diagnosis for each of the 39 subjects. Five of the 16 (31.4%) subjects presenting in the colder months of May, June, and July were either negative or ≤5 JDF U for ICA, but during the rest of the year, only one subject presented with ICA levels of ≤5 JDF U.

There was a significant difference in presentation with low or undetectable ICA levels during the colder months than during the warmer months ($P = 0.028$).

HLA-DQB1 allele frequencies are shown in Table 1 and the genotype frequencies in Table 2. The allele 0302 was identified in 28 subjects (71.8%). Seventeen subjects (43.5%) were heterozygous 0302/0201, with the next most prevalent genotype being homozygous 0302 (20.5%). The two part-Maori subjects (who were sisters) were both typed 0302/0201, and the part-Melanesian subject was homozygous 03032. The distribution of alleles encoding nonaspartic acid (NA) and aspartic acid (A) at position 57 on the peptide chain was as follows: 37 subjects (94.9%) were NA/NA, none were heterozygous NA/A, and 2 (5.1%) were A/A. All seven ICA$^-$ subjects were homozygous for nonaspartate at codon 57.

Only 14 subjects (35.9%) were both ICA$^+$ and susceptibility genotype

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Table 1—*HLA-DQB1 allele frequencies in 39 type I diabetic subjects*

Allele	0302	0201	03032	0501	0602,3	0604,5
Frequency (%)	71.8	51.3	2.6	10.3	2.6	12.8

Allele frequency indicates the percentage of subjects presenting with a given allele. World Health Organization nomenclature is used for the HLA-DQB1 alleles. Of the 17 known alleles, only those shown were present in the 39 subjects.

0302/0201. Twenty-four subjects (61.5%) were both ICA$^+$ and had allele 0302, reflecting the conditional probabilities of 84.6 and 71.2%, respectively. Table 3 shows the ICA and age characteristics of the assigned genotype groups. The probability of being ICA$^+$ was not significantly different between subjects with allele 0302 (groups 1 and 2, 85.7%) and those without 0302 (group 3, 81.8%). There was no correlation between DQB1-defined genetic susceptibility and ICA positivity, and the ICA$^+$ and ICA$^-$ subjects were distributed across the susceptibility classes.

There was no difference in mean age of the subjects with susceptibility typing of groups 1 and 2, but there was a trend for those with nonsusceptibility genotypes (group 3) to be older than subjects with 0302 ($P = 0.056$). No other variables (sex, family history, duration of symptoms, glucose level, or ketones at diagnosis) correlated with DQB1 typing.

CONCLUSIONS— From this study of new cases of type I diabetes, there does not appear to be a simple relationship between HLA-DQB1 genetic typing and presentation with ICA. The allele frequencies described are consistent with reports from other Caucasoid type I diabetic populations (6,8,14,15). Similarly, the prevalence of ICA at 84.6% is in agreement with other studies (18,28,29) and with the rates observed during prior years in Canterbury, New Zealand. Although only 39 individuals were studied, these subjects were both clinically and epidemiologically well defined. Thus, we expected to detect any existing association between ICA and HLA-DQB1 in this cohort of subjects. Overall, 61.5% of the study cohort were positive for both allele 0302 and ICA, but ICA positivity at diagnosis did not vary with genetic susceptibility. The lack of association between these two markers would suggest that variations at the DQB1 locus are not involved in the autoimmune process of type I diabetes. This conclusion among newly diagnosed type I diabetes cases is in accord with other studies. HLA-DR types DR3 and DR4 are more prevalent in diabetic subjects whether they have ICA or not (24), and among high-risk first-degree relatives of type I diabetic subjects included on the Pittsburgh registry (30), the presence of susceptibility DQ heterodimers for type I diabetes was unrelated to production of islet cell antibodies. Thus, as stated by Lipton et al. (30), being genetically at risk for type I diabetes does not imply an association with autoimmunity. The presence of both susceptibility alleles and ICA appears to be necessary for diabetes to occur.

There have been mixed reports regarding whether there is a correlation between ICA positivity and age at onset (18,19,28,29,31). Our results showed no significant correlation with age or sex. The clustering of cases with low ICA levels in the colder months of the year is of interest because ICA$^-$ presentations of type I diabetes could indicate that the diabetes is a result of acute β-cell destruction rather than a chronic autoimmune process. Nonautoimmune β-cell destruction was proposed by us as a possible explanation for the rapid upsurge in type I diabetes incidence rates from 1990 to 1993; however, the study data do not sustain this hypothesis.

The family history data indicate a low incidence of cases with first-degree relatives with type I diabetes of about 10%, comparable to other studies (28). Over these 2 study years with a high incidence rate for type I diabetes, 51.3% of cases were <10 years of age at diagnosis compared with 23.8% during 1982–1989. Genetically susceptible (groups 1 and 2) subjects presented at a younger age than the nonsusceptible subjects (group 3). A Swedish study did not find any such associations between age at diagnosis and DQB1 (32). Whereas a relationship be-

Table 2—*HLA-DQB1 genotypes of 39 type I diabetic subjects*

Genotype	*n* (%)	Designation	Codon 57
Group 1			
0302/0201	17 (43.5)	Most susceptible	NA/NA
Group 2			
0302/0302	8 (20.5)	Susceptible	NA/NA
0302/0501	1 (2.6)	Susceptible	NA/NA
0302/0604,5	2 (5.1)	Susceptible	NA/NA
Group 3			
0201/0201	3 (7.7)	Neutral	NA/NA
0604,5/0604,5	3 (7.7)	Neutral	NA/NA
03032/03032	1 (2.6)	Neutral	A/A
0501/0501	3 (7.7)	Neutral	NA/NA
0602,3/0602,3	1 (2.6)	Protective	A/A

The genotypes are grouped on the basis of risk association with type I diabetes (see DISCUSSION). NA/NA, homozygous nonaspartic acid; A/A, homozygous for aspartic acid residue at codon 57.

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Table 3—*ICA and age characteristics of genotype groups*

Genotype	*n*	Mean age (years)	ICA$^+$	ICA$^-$
Group 1	17	8.3		
0302/0201			14 (82.4)	3 (17.6)
Group 2	11	8.1		
0302/0302			7 (87.5)	1 (12.5)
0302/0501			1 (100)	
0302/0604,5			2 (100)	
Group 3	11	11.8		
0201/0201			2 (66.6)	1 (33.4)
0604,5/0604,5			2 (66.6)	1 (33.4)
03032/03032			1 (100)	
0501/0501			3 (100)	
0602,3/0602,3			1 (100)	

Data are *n* (% of total) unless otherwise indicated.

tween DR4 and younger age of diabetes onset has been described (33), this has not been verified by other studies (18, 34). Relationships between family history and age of onset or between nonketotic presentations and low ICA as reported here may merely be an artifact of small study size.

Thus, in this study spanning 2 years of high type I diabetes incidence rates, no direct relationship was observed between DQB1-defined genetic susceptibility and ICA at diagnosis, suggesting that variations at the DQB1 locus are not linked to the expression of this autoimmune marker of β-cell destruction.

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