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Genetics

Worldwide differences in the incidence of type I diabetes are associated with amino acid variation at position 57 of the HLA-DQ β chain

(genetic marker/gene frequency/autoimmunity/epidemiology/disease susceptibility)

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ABSTRACT **The presence of an amino acid other than aspartic acid at position 57 of the HLA-DQ β chain (non-Asp-57) is highly associated with susceptibility to insulin-dependent diabetes mellitus (IDDM), whereas an aspartic acid at this position (Asp-57) appears to confer resistance to the disease. We hypothesize that the 30-fold difference in IDDM incidence across racial groups and countries is related to variability in the frequency of these alleles. Diabetic and nondiabetic individuals were evaluated in five populations, including those at low, moderate, and high risk. HLA-DQ β genotype distributions among the IDDM case groups were markedly different ($P < 0.001$), as were those among nondiabetic controls ($P < 0.001$). Non-Asp-57 alleles were significantly associated with IDDM in all areas; population-specific odds ratios for non-Asp-57 homozygotes relative to Asp-57 homozygotes ranged from 14 to 111. Relative risk information from the case-control study and population incidence data were combined to estimate genotype-specific incidence rates for the Allegheny County, PA, Caucasians. These rates were used to predict the overall incidence rates in the remaining populations, which were within the 95% confidence intervals of the actual rates established from incidence registries. These results are consistent with the hypothesis that population variation in the distribution of non-Asp-57 alleles may explain much of the geographic variation in IDDM incidence.**

Many chronic diseases exhibit marked geographic variation in incidence (1). Insulin-dependent (type I) diabetes mellitus (IDDM) is one such disorder for which the worldwide patterns of incidence have been well documented (2–4). Age-adjusted IDDM incidence rates are very high in the Scandinavian countries, such as Finland (29.5/100,000 per year) (4), but are extraordinarily low in the Oriental populations, such as China (5) and Japan (4) (0.7/100,000 per year) (Fig. 1). Although the causes of the geographic variation in IDDM incidence are unknown, differences in the distribution of genetic or environmental factors are likely to be related to the variations in disease incidence across populations.

In humans, the HLA region of the short arm of chromosome 6 contains genes of the major histocompatibility complex (MHC), which are highly associated with susceptibility to IDDM (6). We now know that the outer domains of MHC molecules form a cleft in which polypeptides from processed antigens can bind. The shape of the MHC molecule's cleft is determined by critical amino acid segments. Variation in these crucial sequences directly affects the peptide-binding ability of the molecule and permits the proper lodging of some antigens, whereas others are not efficiently bound (7). When properly presented, the helper T-cell recognizes the MHC molecule/polypeptide complex by means of its α/β receptor and becomes activated. The MHC molecule/polypeptide complex is also known to restrict the T-cell clone repertoire of an individual (8). It appears that variation in those critical amino acids of the MHC molecule can influence the thymic selection of T-cell clones by modifying the relationship between the molecule and the processed antigen (9). Our current knowledge of the function of the MHC molecule/polypeptide complex in immune response has provided a logical explanation for the importance of specific amino acid sequences in determining susceptibility to certain autoimmune diseases, such as IDDM.

Recent studies focusing on the HLA-DQ β-chain gene have revealed that the presence of DNA sequences coding for an amino acid other than aspartic acid at the 57th position (non-Asp-57) of this chain is highly associated with susceptibility to IDDM, whereas aspartic acid in this position (Asp-57) appears to confer resistance to the disease (10, 11). We hypothesize that the global patterns of IDDM incidence are associated with population variability in the frequency of the HLA-DQ β alleles. Based on this hypothesis, one would predict that when comparing populations with different IDDM incidences the genotype distributions of both IDDM cases and healthy controls would vary significantly and that the association between IDDM risk and non-Asp-57 alleles would be consistent in all populations. To evaluate these predictions, IDDM cases and nondiabetic controls were randomly selected for HLA-DQ β molecular analyses from five low-, moderate-, and high-risk areas where the incidence of disease was known. By linking the relative risk estimates from the case-control study to the absolute risk of developing the disease, genotype-specific IDDM incidence rates were estimated for Caucasians in Allegheny County, PA. These point estimates were applied to the other four populations to predict the overall IDDM incidence rate for each population. The predicted rates were then compared to those established through the corresponding IDDM registries.

MATERIALS AND METHODS

Study Populations. The populations included in this evaluation were Chinese, Allegheny County Blacks and Caucasians, Norwegians, and Sardinians (Fig. 1). The annual age-adjusted IDDM incidence rate for individuals <15 years of age in these populations and corresponding 95% confidence intervals (CI) were 0.7/100,000 per year (CI = 0.3–1.5) (5), 11.8/100,000 per year (CI = 7.9–17.2) (4), 15.8/100,000 per year (CI = 14.3–17.3) (12), 19.7/100,000 per year (CI =

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; MHC, major histocompatibility complex.

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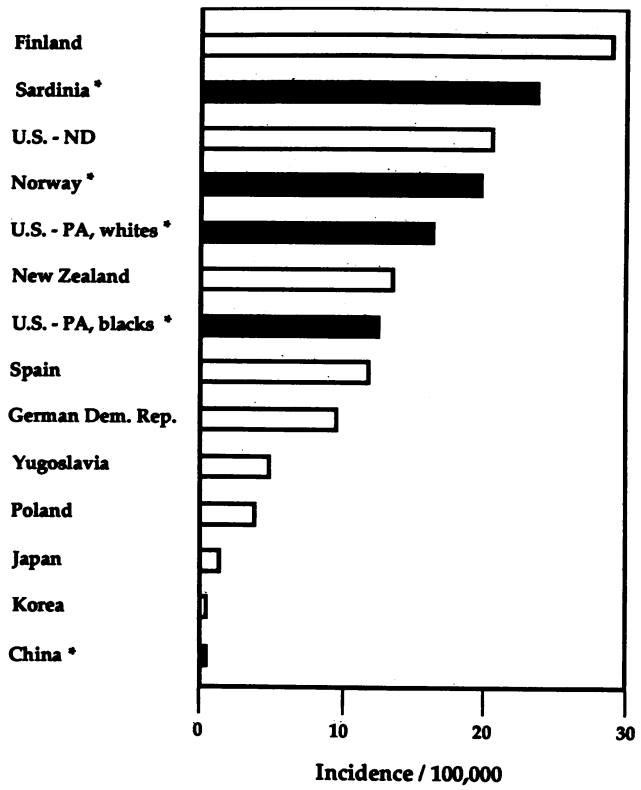

FIG. 1. Geographic differences in age-adjusted annual IDDM incidence rates. Asterisks and black shading indicate populations included in this study.

18.1–21.4) (4), and 24/100,000 per year (CI = 18.5–31.2) (13), respectively.

For each population except the Norwegians, HLA-DQ β molecular analyses were performed in Pittsburgh from blood samples collected from randomly selected IDDM subjects who were <15 years of age at the time of first insulin administration and continued to require insulin treatment. Samples from unrelated nondiabetic controls from each of the populations were also obtained for analyses. We have previously published the HLA-DQ β genotype distributions for Chinese cases and controls (5). A study of the association between non-Asp-57 and IDDM was recently conducted in Norway using laboratory methods and a research design similar to ours (14). These published data are included in our comparative analyses.

Laboratory Methods. Genomic DNA was extracted from peripheral blood lymphocytes of each donor by using standard techniques (15) and subjected to polymerase chain reaction (16) to amplify the second exon of the *DQB1* gene encoding the first external domain of the DQ β chain, as described (11, 17). Dot blot analyses with *DQB1* allele-specific oligonucleotide probes were than carried out. Primers P2 (5'-GATTTCGTGTACCAGTTTAAGG-3') and P4 (5'-CCACCTCGTAGTTGTGTCTGC-3') were used for the amplification. Amplification of 1–2 μg of DNA was carried out with 30 cycles of denaturation at 94°C for 90 sec, annealing of the primers at 37°C for 150 sec, and DNA polymerization at 72°C for 120 sec. In all reactions, the thermostable *Thermus aquaticus* DNA polymerase and a Thermocycler (both from Cetus, Emeryville, CA) were used. A DNA stretch of 241 base pairs was obtained. Dot blot analyses were carried out with 3–5% of the amplified material from each individual diluted in 0.4 M NaOH/25 mM EDTA and applied to Nytran membranes (Schleicher & Schuell) by using a Bio-Dot apparatus (BioRad). The DNA was cross-linked to the membrane by UV irradiation and prehybridized at 42°C. The blots were probed at 42°C overnight with radiolabeled allele-specific oligonucleotides. The filters were then washed with 6× SSC/0.1% SDS (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) to remove any probe that had one or more mismatches with the target sequence. The following oligonucleotide probes were used to identify specific Asp-57 alleles (the indicated temperatures are those required to wash off mismatched probes): *DQw1.2*, 5'-GGCGGCCTGTTGCCGAG-3' [amino acids (aa) 54–59] at 62°C; *DQw1.9*, 5'-GGCGGCCTGACGCCGAG-3' (aa 54–59) at 62°C; *DQw3.1* and *DQw3.3*, 5'-GGCCGCCTGACGC-CGAG-3' (aa 54–59) at 66°C; *DQwBlank*, 5'-GGCGGCT-TGACGCCGAG-3' (aa 54–59) at 60°C. To distinguish *DQw3.1* from *DQw3.3*, the use of an additional probe specific for *DQw3.1* around aa 27 was necessary: *DQw3.1-27*, 5'-CGTGCGTTATGTGACCA-3' (aa 23–29) at 52°C. Probes used to identify non-Asp-57 alleles were *DQw1.1*, 5'-GGCGGCCTGTTGCCGAG-3' (aa 54–59) at 62°C; *DQw1.AZH*, 5'-GGCGGCCTAGCGCCGAG-3' (aa 54–59) at 62°C; *DQw2*, 5'-GCTGGGGCTGCCTG-3' (aa 52–58) at 62°C; *DQw3.2*, 5'-GGCCGCCTGCCGCCGAG-3' (aa 54–59) at 65°C. To overcome the problem of cross-hybridization between *DQw3.1*, *DQw3.2*, and *DQw3.3* and to reduce the time necessary for these determinations, our "digestion" method was frequently used in parallel experiments (17).

Statistical Methods. *Genotype distributions.* The HLA-DQ β genotype distributions among IDDM cases from five populations were compared using a Fisher's exact test for a 3 × 5 table (18). This analysis was repeated to compare the genotype distributions in the five control populations. The genotype distributions in the control populations were tested for Hardy–Weinberg equilibrium by using a χ^2 goodness-of-fit test.

Genotype-specific incidence. The overall incidence of IDDM in a given population can be expressed as a weighted average of the genotype-specific incidence rates, where the weights are the proportions of the population exhibiting each genotype (19). These weights can be estimated from the control data for the population, and the overall incidence rate can be represented by the following equation:

$$R = R_2P_2 + R_1P_1 + R_0P_0, \quad [1]$$

where R_2 = incidence among non-Asp-57/non-Asp-57, R_1 = incidence among non-Asp-57/Asp-57, R_0 = incidence among Asp-57/Asp-57, P_2 = proportion with non-Asp-57/non-Asp-57, P_1 = proportion with non-Asp-57/Asp-57, and P_0 = proportion with Asp-57/Asp-57.

Relationships between the genotype-specific incidence rates can be expressed in terms of relative risks (*RR*). For example, $R_2/R_0 = RR_{20}$ is the risk of IDDM among non-Asp-57 homozygotes relative to the risk among the Asp-57 homozygotes. Similarly, $R_1/R_0 = RR_{10}$ is the risk among heterozygotes relative to that among Asp-57 homozygotes. Each relative risk can be estimated by an odds ratio from a case-control study when the disease is rare (19). We used Woolf's method, as modified by Haldane, for small sample sizes (20). Exact *P* values for tests of homogeneity of odds ratios were also obtained (18).

For known incidence *R*, Eq. 1 becomes a function of the single unknown R_0, the risk for the baseline category, when the estimated population proportions P_2, P_1, and P_0 and the estimated relative risks RR_{20} and RR_{10} are substituted into the equation as follows:

$$R = R_0(RR_{20}P_2 + RR_{10}P_1 + P_0). \quad [2]$$

The equation can be solved for R_0, after which the genotype-specific risks R_1 and R_2 can be estimated from the values of R_0 and the corresponding odds ratio.

Table 1. Distributions of HLA-DQ β genotypes and non-Asp-57 gene frequencies among diabetics and nondiabetic unrelated controls in five populations

Population	Diabetics					Nondiabetics				
	n	N/N	N/A	A/A	*N*	*n*	N/N	N/A	A/A	*N*
Sardinians	30	1.00	0.00	0.00	1.00	60	0.38	0.47	0.15	0.62
Norwegians	52	0.80	0.16	0.04	0.89	187	0.27	0.51	0.22	0.53
U.S. Caucasians	49	0.61	0.39	0.00	0.81	123	0.20	0.46	0.34	0.43
U.S. Blacks	30	0.73	0.27	0.00	0.87	51	0.18	0.37	0.45	0.36
Chinese	18	0.06	0.72	0.22	0.42	25	0.00	0.08	0.92	0.04

n, Number of subjects; N/N, non-Asp-57 homozygosity; N/A, non-Asp-57 heterozygosity; A/A, Asp-57 homozygosity; *N*, non-Asp-57 gene frequency.

The genotype-specific rates obtained for Allegheny County Caucasians were applied to HLA-DQ β genotype distributions for the four independent control populations, and the overall incidence rates were estimated and compared to the published rates for these populations.

RESULTS

Genotype Distributions. The distributions of the HLA-DQ β genotypes for the IDDM cases and nondiabetic controls in the study populations are presented in Table 1. The genotype distributions for the IDDM cases were markedly different in the five populations ($P < 0.001$), as were those for the controls ($P < 0.001$). The observed genotype distributions for the control populations were not significantly different from the expected proportions under the assumption of Hardy–Weinberg equilibrium. This indicated that the control samples were representative of the general population at risk for developing the disease.

The higher observed prevalence of heterozygotes and Asp-57 homozygotes among IDDM cases in the lower risk populations reflected their distribution among nondiabetic controls, where the frequencies of these genotypes were also higher. It was anticipated that the genotype distribution among IDDM cases in a given area or racial group would reflect the proportions in the general population.

Genotype-Specific Incidence. The estimated relative risks associated with the presence of one or more non-Asp-57 alleles are presented in Table 2. The odds ratios for non-Asp-57 homozygosity relative to Asp-57 homozygosity were all highly significant. The exact test for homogeneity was nonsignificant ($P = 0.26$). The odds ratios for one non-Asp-57 allele compared to Asp-57 homozygotes were statistically significant for Allegheny County Caucasians and Blacks and the Chinese and nonsignificantly elevated for the Norwegians. None of the IDDM cases from Sardinia were heterozygous, so the odds ratio was not estimated. There was evidence for heterogeneity of these odds ratios ($P = 0.03$), primarily due to the Norwegian data.

The estimated genotype-specific IDDM incidence rates for Caucasians in Allegheny County were highest among non-Asp-57 homozygotes (47.6/100,000 per year), intermediate for heterozygous individuals (13.0/100,000 per year), and lowest for Asp-57 homozygotes (0.45/100,000 per year). These findings are suggestive of a dose–response relationship between IDDM susceptibility and this genetic marker.

Table 2. Odds ratios for IDDM associated with non-Asp-57 homozygosity (N/N) and heterozygosity (N/A) relative to Asp-57 homozygosity (A/A)

Population	Odds ratio	
	N/N vs. A/A	N/A vs. A/A
Sardinians	24.7*	NE
Norwegians	13.7*	1.5†
U.S. Caucasians	105.8*	28.8*
U.S. Blacks	111.3*	20.5*
Chinese	15.7‡	28.2*

NE, Not estimated.
*, $P < 0.0001$; †, $P > 0.05$; ‡, $P < 0.05$.

Because the statistical properties of the estimated genotype-specific rates are currently unknown, we addressed the similarities of the genotype-specific incidence rates across populations indirectly. The genotype-specific rates for Allegheny County Caucasians were used as point estimates and applied to the HLA-DQ β genotype distributions among the Chinese, Allegheny County Blacks, Norwegian, and Sardinian controls (Table 1) to predict the overall age-adjusted incidence for each population. Each predicted rate (1.45/100,000 per year for the Chinese, 13.6/100,000 per year for the Blacks, 19.6/100,000 per year for the Norwegians, and 24.3/100,000 per year for the Sardinians) fell within a 95% confidence interval for the corresponding rate established through an IDDM incidence registry (Fig. 2). These findings support our hypothesis that the geographic and racial variations in IDDM incidence are directly related to different genetic backgrounds in each population.

DISCUSSION

With advances in molecular biology, host susceptibility markers for IDDM are now being studied at the DNA level, focusing on molecular variation in the region encoding amino acid 57 of the HLA-DQ β chain (10, 11). There appears to be a hierarchical scale of susceptibility within the group of

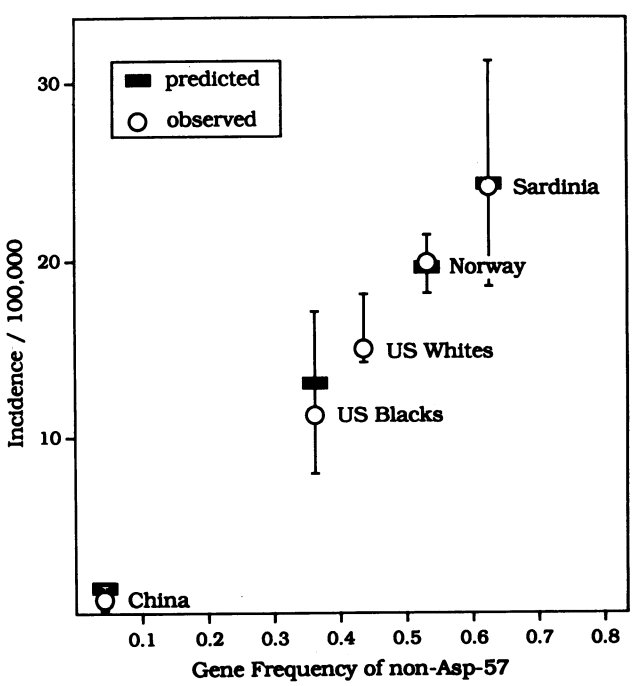

FIG. 2. Association between actual and predicted age-adjusted annual IDDM incidence rates. The 95% confidence intervals are indicated by the bars associated with each observed incidence value.

non-Asp-57 (and Asp-57) alleles, as the susceptibility (or protection) conferred by the individual alleles is not equally strong (10). Thus, it is likely that other amino acids of the DQ α and β chains (10, 21–23) or specific combinations of DQ β and DR β sequences in the same haplotype may further contribute to IDDM susceptibility (24). These are possible explanations for the observed increase risk for DR3/DR4 heterozygotes, compared to DR3 or DR4 homozygous individuals. In addition, the *DQA1*0301* allele, which distinguishes Caucasian from Black DR7, non-Asp-57 haplotypes, may account for the lack of DR7-associated susceptibility among Caucasians (23). What is critical, however, for our study is that the class of alleles defined by non-Asp-57 represents the strongest single marker of susceptibility to IDDM that has yet been identified.

Although studies of the association between non-Asp-57 and IDDM have been previously conducted on small samples in other ethnic groups (5, 22–26), the current report represents, to our knowledge, the first comparative population-based case-control analyses of non-Asp-57 and IDDM susceptibility. Data from five populations were evaluated to determine the contribution of the non-Asp-57 allele to the incidence of the disease within populations and between racial groups and countries. These results indicate that (*i*) between populations, there are major geographic and racial differences in the distribution of non-Asp-57 among both IDDM cases and nondiabetic controls, (*ii*) within each population studied, the association between non-Asp-57 and IDDM is consistently strong, as evidenced by highly significant odds ratios, and (*iii*) there is close agreement between the observed IDDM incidence rates and the ones predicted from the non-Asp-57 genotype frequency in each population.

These results indicate that the effects of other genes, environmental factors, or their interaction are likely to be uniform across populations and/or minor in comparison to the contribution of the non-Asp-57 allele in determining the global differences in incidence. If genotype-specific risks are truly uniform across populations, it will also be possible to approximate the incidence of IDDM in a new population by simply testing for non-Asp-57 in a representative sample of unrelated nondiabetic controls from that population.

Although it appears that genetic differences across populations may be responsible for the geographic patterns of IDDM incidence, environmental and/or genetic factors, other than Asp-57 and non-Asp-57, clearly play a role in the etiology of the disease. For example, many non-Asp-57 homozygous individuals in the population do not develop the disease, and the diabetes concordance rates among monozygotic twins is typically low, ranging from 30% to 50% (27). In addition, "epidemics" of IDDM have been reported (28). Possible explanations for these findings include (*i*) the presence or absence of other IDDM susceptibility genes (29), (*ii*) variation in exposure to environmental triggers, and (*iii*) immunological differences. The latter provides the most likely explanation for discordant monozygotic twins and takes into consideration such matters as the somatically rearranged T-cell receptor genes and the resultant different T-cell repertoires present in these individuals.

Through the development of IDDM incidence registries in populations across the world, standardized data regarding the geographic and racial patterns of incidence are now available (2, 4). A global population-based immunogenetic study is, therefore, necessary to confirm these results with larger samples. For example, there are conflicting published data concerning the frequency of non-Asp-57 in the Japanese. While one Japanese investigation is entirely consistent with our predictions (25), other studies reported considerably different prevalence values of non-Asp-57 among nondiabetic controls (22, 26). Although the reasons for such inconsistencies are unclear, it is likely that methodological differences are a contributing factor.

International immunogenetic studies must be based upon standardized epidemiologic methods, which will ensure the selection of comparable samples of IDDM cases and nondiabetic controls in areas where the incidence of disease is known. Such investigations will also provide a link between the relative and absolute risk of developing the disease across populations, as illustrated by our estimates of the genotype-specific incidence rates. In Allegheny County, we estimate that $\approx$1 in every 2100 Caucasian children who are non-Asp-57 homozygous will become diabetic each year, whereas only 1 in every 7700 heterozygous individuals will develop IDDM annually. These estimates translate into cumulative risks of developing IDDM through age 20 years of 0.9% and 0.3% for non-Asp-57 homozygotes and heterozygotes, respectively. The risk for Asp-57 homozygotes was estimated to be even lower: only 1 in every 222,000 children will become diabetic each year.

Many chronic diseases exhibit marked geographic variation in incidence, which has frequently been attributed to environmental differences across populations (1). This investigation stresses the importance of large genetic variations between racial groups and countries. One may speculate that differences in susceptibility genes for other chronic diseases exist and contribute significantly to the geographic patterns of incidence of these disorders. The development of an interface between epidemiology and molecular biology represents a research approach that will contribute to our better understanding of the relationships between specific risk factors and the etiology of chronic diseases.

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