Polymorphism in the 5′ Flanking Region of the Human Insulin Gene

Relationships with Noninsulin-dependent Diabetes Mellitus, Glucose and Insulin Concentrations, and Diabetes Treatment in the Pima Indians

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Abstract. Variations in DNA sequences flanking the insulin gene were studied in relation to noninsulin-dependent diabetes mellitus (NIDDM) in 87 unrelated Pima Indians at least 35 yr of age. DNA was isolated from nuclei of peripheral blood leukocytes and digested with restriction endonucleases. Less variation in this region was found in Pima Indians than in other racial groups previously studied. Only two classes of alleles (classes 1 and 3) were found, and there was virtually no variation within classes. At least one class 3 allele was found in 47% of the 38 nondiabetic subjects and in 37% of the 49 with NIDDM (odds ratio = 0.65, P = 0.4, 95% confidence interval for the odds ratio = 0.25 to 1.67). Homozygosity for class 3 alleles, however, was found only in diabetics. There were no differences according to genotype in obesity, fasting or postload glucose or insulin concentrations, or in the relationships between insulin and glucose concentrations. 61% (11/18) of the diabetics with a class 3 allele were receiving drug treatment for diabetes compared with only 26% (8/31) of diabetics without a class 3 allele (P = 0.03). The insulin gene polymorphism probably plays no important role in the genesis of NIDDM in Pima Indians, nor does it influence the glucose or insulin concentrations or their relationship to each other, but the class 3 allele, especially when homozygous in this population, may influence the severity of the disease as indicated by need for drug treatment.

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Introduction

Variations in DNA sequences flanking the insulin gene have been described (1, 2). This polymorphic region (locus) is located about 500 base pairs before the transcription initiation site for proinsulin messenger (m)RNA. The proximity of this variable region suggested that it might affect expression of the insulin gene.

Rotwein et al. (3) described the relationship between diabetes and a set of alleles (class 3 alleles in the terminology of the present paper) containing ~1.6 kilobase pairs of extra DNA compared with the most common alleles (class 1). In a racially mixed group, consisting of Caucasians, American blacks, and Pima Indians, the frequency of class 3 alleles was found to be significantly more common in subjects with noninsulin-dependent diabetes mellitus (NIDDM)[1] than in subjects with insulin-dependent diabetes mellitus (IDDM) or in subjects without diabetes (3). Among Danish subjects, Owerbach and Nerup (4) reported class 3 (U in their terminology) alleles to be significantly more frequent in NIDDM than in IDDM. They subsequently found class 3 alleles to be significantly more common in NIDDM than in those with impaired glucose tolerance or in those with normal glycosylated hemoglobin and fasting glucose concentrations (5). Among the nondiabetic members (including unrelated spouses) of a large family containing several persons with IDDM, there was no association of class 3 alleles with fasting glucose, insulin, or C-peptide concentrations. Those with one or two class 3 alleles,

1. *Abbreviations used in this paper:* IDDM, insulin-dependent diabetes mellitus; NIDDM, noninsulin-dependent diabetes mellitus.

however, had significantly higher levels of glycosylated hemoglobin (6). Subsequent studies did not demonstrate associations of class 3 alleles with NIDDM in Micronesians in Nauru (7) nor linkage with diabetes in families with NIDDM in young people (8, 9). Bell et al. (10) recently reported class 1 alleles to be more common in IDDM than in nondiabetics.

We recognized the possibility of racial differences both in the frequencies of DNA polymorphisms and in susceptibility to NIDDM, and conducted a further study of Caucasian, black, and Pima Indian subjects that suggested an association between class 3 alleles and NIDDM in each of the three groups (11). Although the relationship was not strong enough in any group alone to be statistically significant, the data were consistent with the same degree of relationship in all three groups, and the association between class 3 alleles and NIDDM was statistically significant in a test maintaining stratification by race. Nevertheless, there appeared to be racial differences in the strength of the association. The class 3 allele frequency was higher in blacks than in Caucasians or Pimas. This difference was found among nondiabetics and especially among those with NIDDM. Further studies within each group were thus indicated. In this paper we report results of additional studies conducted in Pima Indians.

Methods

Subjects and clinical measures. Subjects were participants in the National Institutes of Health longitudinal population study in the Gila River Indian Community of Arizona (12, 13). A total of 115 individuals (mostly of the Pima Indian tribe) were evaluated for insulin gene polymorphism, but for the purposes of determining the relationship with diabetes, only 87 subjects were included in this report. They were of at least half Pima Indian ancestry, at least 35 yr of age at their last examinations, and unrelated (i.e., none of those included was a first degree relative [parent, sibling, or child] of any other). Whereas the population study has been conducted continuously since 1965, sampling for the DNA studies, conducted from 1980 to 1983, was intermittent. The scheduling of samples for DNA studies was based on laboratory workloads and was independent of the clinical status of the subjects. Some of the subjects reported here were included in previous reports (3, 11).

The presence of diabetes was established at the most recent biennial examination by means of a modified glucose tolerance test interpreted by World Health Organization (WHO) criteria (14), or by a previous diagnosis (see below). After an overnight fast, venous plasma samples were obtained for glucose determination before and 2 h after ingestion of a 75-g carbohydrate load (Glucola, Ames Co., Elkhart, IN). Diabetes was diagnosed when the 2-h glucose was at least 200 mg/dl (11.1 mmol/liter). The WHO criteria also allow for a diagnosis based on a fasting plasma glucose concentration of at least 140 mg/dl (7.78 mmol/liter), but all subjects with this degree of fasting hyperglycemia also had 2-h glucose concentrations > 200 mg/dl. Two subjects whose latest glucose tolerance tests did not meet these diagnostic criteria were also included with the diabetics. One had had 2-h plasma glucose concentrations of >200 mg/dl on two previous occasions; one had had a casual glucose concentration of >500 mg/dl and had been treated with insulin. All other subjects, including those with impaired glucose tolerance by the WHO criteria, were considered nondiabetic. Analyses in which the group with impaired glucose tolerance was considered separately did not alter the conclusions and are not presented. For the six individuals not examined fasting, the classification was made on the basis of the 2-h plasma glucose alone.

Other clinical and laboratory data were obtained at the time of the modified glucose tolerance test. Drug treatment for diabetes was classified according to what the patient reported taking within the 4 d preceding the examination. Fasting and 2-h serum insulin concentrations were determined using the Herbert modification of the Berson and Yalow radioimmunoassay (15). Obesity was estimated by the body mass index (weight per height^2 in kilograms per meter^2). Retinopathy was determined by direct ophthalmoscopy after pupillary dilatation as previously described (16). Any of the following lesions, occurring in any number and in either eye, was considered diabetic retinopathy: microaneurysms, retinal hemorrhages of any size, neovascularization, or preretinal hemorrhage (17). A resting 12-lead electrocardiogram interpreted by an independent reader was classified according to the Whitehall criteria (18). Fasting plasma triglycerides were measured in 33 of the subjects as part of another study; the methods and results of which are reported elsewhere (19).

DNA studies. DNA was isolated from nuclei of peripheral blood leucocytes and digested with restriction endonucleases as described (3). The terminology suggested by Bell et al. (10) is used to describe the classes of alleles found. The variable region lies between ~350 and 850 base pairs (bp) 5′ (upstream) from the transcription initiation site. Thus, the average size of the most common class 1 allele is 570 bp. Class 1 alleles were previously called common alleles by us (3, 11) and L alleles by Owerbach et al. (4–6, 9). There is also variation in size within class 1 alleles (3, 10), presumably due to different numbers of tandemly repeating 14–15 bp oligonucleotide units (2). Class 3 alleles, of average size 2,170 bp, were called inserts (3, 11) or U alleles (4–6, 9) in previous reports. No class 2 alleles were found.

Statistical analyses. The associations of the two classes of the DNA polymorphism and categorical variables such as presence of diabetes or complications were assessed by standard contingency table analyses with chi-square or exact tests. The confidence interval for the odds ratio was computed by the exact (Fisher) method of Rothman and Boice (20). The distributions of continuous variables, such as glucose and insulin, were compared between the two classes with t tests for means and F tests for variances. To test whether the relationship of plasma glucose and serum insulin concentrations differed between classes, the well known nonlinear relationships of glucose and insulin concentrations (fasting or postload) were described with polynomial regression equations. The effects of the DNA classes on these relationships were tested by including class indicator variables in the models (i.e., by analysis of covariance models (21)). The effects of other variables, such as age, sex, and body mass index, were controlled by including them in the regression equation.

Results

Insulin gene polymorphism in the Pimas. Virtually no variation in class 1 alleles was found in Pimas in that only one individual had an allele that was 100–150 bp smaller than the other class 1 alleles. Class 3 alleles have been found to vary in size in Caucasians and blacks to as large as 8,000 bp in our studies (3, 11). There was remarkable consistency in size of class 3 alleles in the Pimas. Of the 230 alleles examined, only one

was larger (~4,000 bp). The frequency of class 1 alleles was 0.76 and of class 3 alleles was 0.24 in the entire sample of 115 Indians. No other classes were found. In the 87 unrelated Pimas at least 35 yr of age, the class 1 and 3 allele frequencies were 0.77 and 0.23, respectively.

Relationship between class 3 alleles and NIDDM. The distribution of DNA classes in diabetic and nondiabetic subjects is shown in Table I for the 87 unrelated Pima Indians at least 35 yr old. There was no significant difference in the distributions according to diabetes, with 47% of the nondiabetics and 37% of the diabetics having at least one class 3 allele (odds ratio = 0.65, $P = 0.4$, 95% confidence interval for the odds ratio of 0.25–1.67). Homozygosity for class 3 alleles was found only in diabetics, but with only four homozygous individuals found; this association with diabetes was consistent with chance.

To test for subtle differences in glucose tolerance not detected by the simple classification of diabetes used above, the distributions of fasting and 2-h plasma glucose concentrations, as well as the distributions of fasting and 2-h serum insulin concentrations, age at examination, age at diagnosis of diabetes, body mass index, and fasting plasma triglyceride concentrations were compared. Table II shows that none of the means and variances of these variables differed significantly between those with and without a class 3 allele. Furthermore, when examined within nondiabetic or diabetic subjects, none of these differences was statistically significant (not shown).

The relationship between fasting plasma glucose and fasting serum insulin concentrations is shown in Fig. 1. In the range of normal glucose, there was a positive association between glucose and insulin concentrations, but above glucose concentrations of ~140 mg/dl, there was a slight decline in insulin concentrations with increasing hyperglycemia, as reported previously in Pima Indians (22, 23) and in several other populations (24–28). Because the serum insulin concentration is uninterpretable in insulin-treated subjects, the glucose and insulin concentrations of these seven subjects are not shown. They were all severely hyperglycemic, however, with glucose concentrations ranging from 201 to 318 mg/dl fasting and 308 to 532 mg/dl 2-h postload. The glucose and insulin concentrations for subjects treated with oral hypoglycemic agents are specifically identified on the graph. As can be seen by inspection, the subjects with class 3 alleles fell on the same curve as those with only the more common class 1 alleles. This conclusion was confirmed by fitting a quadratic regression equation to the relationship. (Higher order polynomial terms did not contribute significantly to the model.) A term for the presence of class 3 alleles did not add significantly to the model, indicating no significant difference in the fasting insulin-glucose relationships between subjects with and without class 3 alleles. When other variables related to glucose and insulin concentrations, such as age, sex, and body mass index, were included in the regression equations, there was still no significant effect of the DNA class. A similar analysis revealed no difference in the 2-h insulin-glucose relationship between DNA classes (Fig. 2).

All four subjects homozygous for class 3 alleles were severely diabetic, with high fasting and 2-h plasma glucose concentrations. Two were treated with oral hypoglycemic agents (Figs. 1 and 2) and two with insulin.

Treatment, retinopathy, and electrocardiographic findings are shown in diabetic and nondiabetic subjects in Table III. Diabetics with at least one class 3 allele were more likely to be treated with drugs (oral agents or insulin) than were diabetics with only class 1 alleles. 61% of the diabetics with a class 3 allele received drug treatment for diabetes compared with only 26% of diabetics without a class 3 allele ($P = 0.03$). There were no significant differences by class in prevalence of retinopathy or abnormal electrocardiogram by Whitehall criteria. Retinopathy was observed in only one nondiabetic subject, in whom a single small retinal hemorrhage was seen. She had normal glucose tolerance at the time, but had had two previous examinations in which she had impaired glucose tolerance by WHO criteria (14). There was no other known cause of her retinopathy.

Table I. Genotype Frequencies According to Diabetic Status in Unrelated Pima Indians ≥ 35 Yr of Age

	Genotype					
	1,1		1,3		3,3	
Diabetes	No.	Frequency	No.	Frequency	No.	Frequency
No	20	0.53	18	0.47	0	0.00
Yes	31	0.63	14	0.29	4	0.08

Discussion

Differences in alleles in the 5′ flanking region of the insulin gene appear to play no direct role in causing NIDDM in the Pima Indians, nor in other aspects of physiology we have measured, such as serum insulin concentrations, obesity, or vascular complications of diabetes. Class 3 alleles were, however, associated with disease severity, as indicated by need for drug treatment. Others have reported NIDDM to be associated with class 3 alleles in Danes (4), but not in Nauruans (7). Bell et al. (10) reported an association of class 1 alleles with IDDM in Caucasians, but had insufficient sample sizes for assessing associations of IDDM in American blacks or other racial groups (10). In contrast with some of the previous reports in Caucasians, they found the class 3 allele frequency to be lower in NIDDM than in nondiabetics. In American blacks there was no significant difference in allele frequency between nondiabetics and those with NIDDM. Thus the findings of Bell et al. (10) with respect to NIDDM in Caucasians and in American blacks are similar to the present findings in Pima Indians.

In a paper describing the methodology used in the present

Table II. Clinical Characteristics of Pima Indians According to Genotype at the Polymorphic Insulin-gene Flanking Region

Genotype	1,1			1,3 or 3,3		
	n	Mean	SD	n	Mean	SD
Age at examination (*yr*)	51	44.5	5.4	36	44.5	6.3
Age at diagnosis of diabetes* (*yr*)	31	39.4	5.8	18	40.6	7.5
Body mass index (kg/m^2)	51	32.2	5.9	36	33.4	6.6
Log fasting plasma glucose‡ (*mg/dl*)	49	2.17 (149)	0.19	32	2.17 (147)	0.19
Log 2-h plasma glucose‡ (*mg/dl*)	51	2.36 (229)	0.26	36	2.33 (214)	0.27
Log fasting serum insulin‡§ ($\mu U/ml$)	46	1.55 (35)	0.29	28	1.58 (38)	0.22
Log 2-h serum insulin‡§ ($\mu U/ml$)	48	2.04 (110)	0.44	32	2.12 (130)	0.32
Log fasting plasma triglycerides‡§ (*mg/dl*)	23	2.21 (163)	0.29	10	2.21 (163)	0.20

There were no significant differences in mean or SD of any of these variables between those with or without a class 3 allele. * Applies only to diabetic subjects (note smaller number of subjects). ‡ Logs (base 10) of these variables were analyzed because of skewed distributions. The geometric means (antilogs of means on the log scale) are given in parentheses. § Excluding insulin-treated subjects.

study, Rotwein et al. reported an association of class 3 alleles with NIDDM in a racially mixed group consisting of Caucasian and black subjects from St. Louis and Pima Indians from Arizona (3). Because mixing racial groups could confound the association, we then performed further studies in these three racial groups and reported the findings within each race.

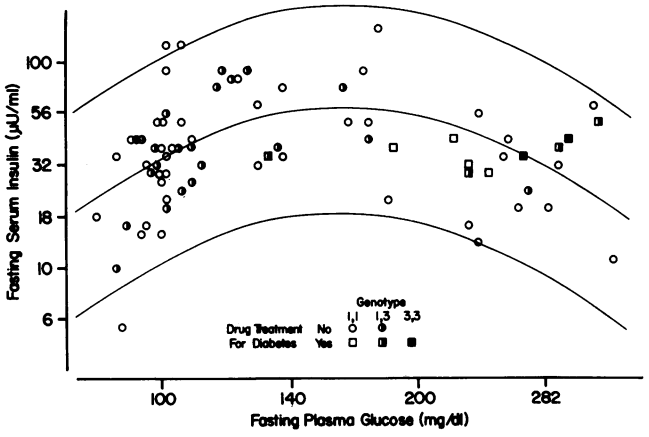

Figure 1. Relationship of fasting serum insulin with fasting plasma glucose concentrations according to genotype at the 5′ flanking region of the human insulin gene. 80 subjects treated without drugs or with oral hypoglycemic agents are shown, but the 7 treated with insulin are omitted. The curves show the quadratic regression equation for fasting insulin as a function of fasting glucose (center) and the 95% confidence limits for individual values (top and bottom). Note that the genotype has no apparent effect on the insulin-glucose relationship.

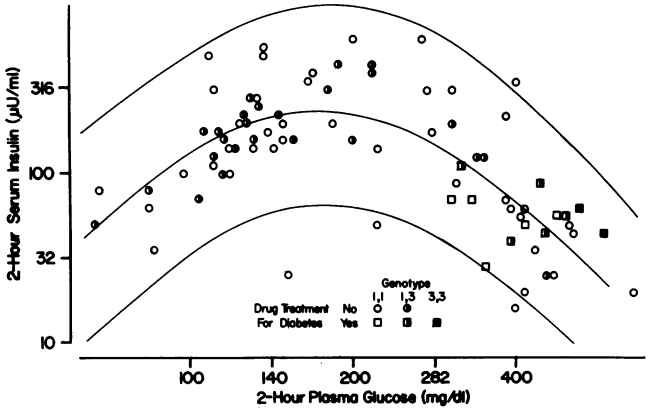

Figure 2. Relationship of 2-h serum insulin with 2-h plasma glucose concentrations according to genotype at the 5′ flanking region of the human insulin gene. The symbols and lines are as in Fig. 1. 80 subjects are shown. There were 42 diabetics, of whom 40 had 2-h glucose concentrations of at least 200 mg/dl, and two had 2-h glucoses under 200 mg/dl but previous diagnoses. The remaining 38 subjects were nondiabetic. Seven insulin-treated subjects are not shown. Note that the genotype has no apparent effect on the insulin-glucose relationship.

Table III. Treatment, Retinopathy, and Electrocardiograms According to Diabetes and the Genotype at the Polymorphic Insulin-gene Flanking Region

	Nondiabetic		Diabetic	
Genotype	1,1	1,3 or 3,3	1,1	1,3 or 3,3
	(n = 20)	(n = 18)	(n = 31)	(n = 18)
Drug treatment				
No	100	100	74	39*
Yes	0	0	26	61
Oral hypoglycemic			(16)	(39)
Insulin			(10)	(22)
Retinopathy	5	0	13	22
Abnormal electrocardiogram	10	22	13	6

In each column the percent of subjects with each characteristic is shown.
* Among diabetics, combining the two drug treatment groups, drug use was significantly more frequent among patients with at least one class 3 allele (Fisher's exact test, $P = 0.03$).

Although the association was not statistically significant in any group considered alone, the finding in all three groups was that NIDDM was associated with class 3 alleles. A statistical analysis which summarized the findings in all groups while maintaining the group-specific comparisons found the overall association to be statistically significant ($P < 0.05$) without significant heterogeneity of the association between the groups. That is, the data were compatible with the same degree of positive association being present within each racial group, even though no group alone was large enough to demonstrate a significant association. It was noted, however, that, although there was no statistically significant heterogeneity in the associations, the observed association was much stronger in blacks (odds ratio = 2.9, comparing presence or absence of a class 3 allele and NIDDM or no diabetes) than in Caucasians (odds ratio = 1.4) or in Pima Indians (odds ratio = 1.5), and that class 3 alleles were more frequent in black subjects. These findings suggested racial differences which the study was not large enough to detect. In view of the present findings and the lack of an association with NIDDM in Caucasians (10) and Nauruans (7), it appears that with the possible exception of black subjects, class 3 alleles do not directly predispose a person to NIDDM. The situation in black Americans is confused by their racial admixture. American blacks have an estimated 10–30% admixture with Caucasian genes (29). Because the class 3 allele frequency is higher in blacks than in Caucasians, and American blacks are at greater risk of NIDDM (30), an association between class 3 alleles and diabetes could occur in a group of mixed African and Caucasian heritage even if the increased susceptibility to diabetes of blacks was due to genetic factors distinct from the polymorphic region of the insulin gene. Thus it is possible that the association of NIDDM and class 3 alleles in black Americans is confounded by racial admixture rather than reflecting direct causality.

In the present study, we found no relationship between class 3 alleles and diabetes, serum insulin concentrations, or diabetes complications. Thus it is unlikely that class 3 alleles play a role in causing diabetes in this population. This finding does not indicate, however, whether the insulin gene might be linked to a major diabetes-susceptibility gene. Such linkage cannot be determined by studies such as the present one which examine samples of unrelated persons in a population, but will require testing of individuals within pedigrees.

The association of class 3 alleles with atherosclerosis reported by Mandrup-Poulsen et al. (31) was not confirmed in the present study. There was no association with abnormal resting electrocardiograms in either nondiabetic or diabetic Pima Indians. Our study did not provide a powerful test of this hypothesis, however, as the resting electrocardiogram is not as sensitive an indicator of coronary artery disease as is the coronary angiography used by Mandrup-Poulsen et al. It is not clear, however, that the control group used in that study, consisting of subjects without atherosclerosis who had undergone coronary angiography for unspecified indications, was representative of a healthy population.

The present study was not designed to assess the association between class 3 alleles and hypertriglyceridemia, but because of the report of Jowett et al. (32) that class 3 alleles were more common in diabetics with hypertriglyceridemia than in nondiabetics with or without hypertriglyceridemia, we investigated this possibility in 33 subjects whose lipids had been measured in a separate study (19). In this subgroup, there was no association of the class 3 allele with plasma triglyceride concentration (Table II) or with cholesterol or any of the lipoprotein fractions (not shown). We were, thus, unable to confirm Jowett's finding in this sample.

Although the presence of at least one class 3 allele was not associated with diabetes in this study, it is remarkable that of the four subjects homozygous for the class 3 allele, not only did all have diabetes, but also in each the disease was sufficiently severe to require treatment with drugs. Thus it is possible that in the Pimas the class 3 allele acts as a recessive diabetes-susceptibility gene, i.e., it is important only when both alleles are present. Despite this suggestion from the present findings, homozygosity for class 3 alleles does not inevitably lead to NIDDM, as several nondiabetic individuals of other racial groups have been found to be homozygous for class 3 alleles (4–6, 10, unpublished observations).

Diabetics with the class 3 allele were more than twice as likely to have their diabetes treated with drugs as were diabetics with only class 1 alleles. Caution must be exercised in interpreting this finding, as many associations were examined, and an association with mode of treatment had not been previously postulated. Hence, it must be considered tentative. Even though many of the subjects were insulin-treated, they should not be considered insulin-dependent (i.e., to have IDDM).

Probably all insulin-treated diabetic Pima Indians have NIDDM by virtue of an absence of islet cell antibodies, ketosis resistance, and discontinuity in insulin treatment (33, 34).

We conclude that the insulin gene polymorphism probably plays no important role in the genesis of NIDDM in Pima Indians, nor does it influence the glucose or insulin concentrations or their relationship to each other, but the class 3 allele, especially when homozygous, may influence the severity of the disease as indicated by need for drug treatment.

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