Genetic, Immunologic, and Environmental Heterogeneity of IDDM

Incidence and 12-Mo Follow-Up of an Italian Population

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SUMMARY

The 1-yr incidence of insulin-dependent diabetes mellitus (IDDM) in a population of the Piedmont and Aosta Valley area of Italy was recorded. Anti-virus antibodies (e.g., Coxsackie B_{1-6}, mumps, cytomegalovirus), islet cell antibodies (ICAs), and HLA-A, -B, -C, and -DR were determined in 74 IDDM patients (38 males, 36 females) and in controls. Total IDDM incidence was 5.0/100,000, and the incidence for those <20 yr of age was 11.6/100,000. Anti-virus antibody frequency was not different in IDDM patients and controls. ICAs were present in 58% of IDDM patients at onset and in 30% after 12 mo, and complement-fixing ICAs were found in 39 and 17%, respectively. IDDM was significantly and positively associated with DR3/DR4 and negatively associated with DR2 and DR5. ICA frequency was significantly higher in DR3/DR4 heterozygote patients than in patients without DR3 and DR4. These results suggest that in this IDDM population *1*) viral etiology is not evident, *2*) ICAs offer only a partial pathogenetic explanation, and *3*) genetic and immunologic heterogeneity is evident. *Diabetes* 36:859–63, 1987

The epidemiologic, genetic, and environmental features of insulin-dependent diabetes mellitus (IDDM) have been described for various populations by several investigators (1–8). Little information, however, is available concerning the Italian population. The incidence of IDDM (9), its immunological component (10), genetic aspects (11), and the features of new cases with a follow-up over a fixed period have been examined in an unsystematic manner for several areas in Italy. This situation is primarily attributable to the lack of a national IDDM register. Evaluation of the genetic aspects (DR antigens in particular) of IDDM is of special interest in view of its possible heterogeneous etiology and pathogenesis (4).

This study was carried out by the Piedmont and Aosta Valley section of the Italian Diabetology Society with the aim of reporting the genetic, immunologic, and demographic features in an IDDM cohort representing all new cases over 1 yr in a geographically defined population.

MATERIALS AND METHODS

This study was conducted in the Piedmont and Aosta Valley area of Italy. During the study the catchment basin consisted of 1,491,068 people, of whom 371,844 were <20 yr old. The cases of IDDM were collected between 2 February 1981 and 31 January 1982. One month before the beginning of the study, all diabetologists, pediatricians, and internists working in the hospitals and diabetes centers of the study area were asked to fill in a longitudinal data card (information on patient diagnosis and quarterly check-ups for the 1st yr of the disease) and send serum samples to the data collection centers. At the end of this period, all records were checked. None of the recorded cases of area residents was missed; cases of nonresidents admitted to area hospitals at clinical onset of diabetes and cases admitted to hospitals outside the area and then referred to a center in the area were not included.

Data collected at diagnosis of IDDM were address, age, sex, onset symptoms, blood glucose, and ketonuria. By National Diabetes Data Group (NDDG; 12) criteria, two subjects aged 64 and 71 yr presenting spontaneous hyperglycemia but not ketonuria (absence of acetoacetic acid and/or β-hydroxybutyric acid) before insulin therapy were not included. Although treated with insulin, these two subjects became non–insulin dependent. Anti-virus antibodies were determined at diagnosis and after 1 mo. Direct islet cell antibodies (ICAs) and complement-fixing islet cell antibodies (CF-ICAs) were measured at onset and quarterly. IDDM patients were typed for HLA-A, -B, -C, and -DR. For ~50% of

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TABLE 1
Month of onset, sex, age, and number of fourfold or greater rises of anti-virus antibodies in 74 subjects with insulin-dependent diabetes mellitus (IDDM) and in 52 sibling controls (SC)

Month of onset	*n*	IDDM subjects (%)	M/F	Age (yr)	Anti-virus–antibody positivity							
					Coxsackie							
					B_1	B_2	B_3	B_4	B_5	B_6	Mumps	Cytomegalovirus
January	5	6.7	3/2	18.2 ± 6.8				1	1			
February	14	18.9	7/7	18.2 ± 17.5				1				
March	9	12.2	3/6	15.7 ± 5.9		1			1		1	
April	4	5.4	1/3	18.5 ± 5.8			1				1	
May	8	10.8	4/4	17.2 ± 15.3								
June	3	4.0	3/0	28.0 ± 7.5								
July	6	8.2	2/4	32.8 ± 23.0		1						
August	1	1.3	0/1	5.0 ± 0				1				
September	11	14.8	8/3	20.3 ± 14.0							1	
October	4	5.4	1/3	13.5 ± 7.8		1		1				
November	6	8.2	3/3	17.6 ± 8.4							1	
December	3	4.0	3/0	23.3 ± 4.7								
Total												
IDDM	74		38/36	19.4 ± 13.6	0	3	1	4	2	0	4	0
SC	52		28/24	21.3 ± 11.4	0	1	0	2	0	0	2	0

Values for age are means ± SD.

the patients the parents were also typed. This allowed us in some cases to deduce the HLA genotype of the proband.

Controls. Fifty-two healthy siblings were used as controls for the presence of anti-virus antibody and ICA, together with 126 blood donors for ICA and 526 healthy subjects for HLA typing. Informed consent was obtained from all patients and controls and from their parents when necessary.

Anti-virus antibodies. Anti-Coxsackie virus B_{1-6} antibodies were measured by the replicate-neutralization test with viral strains (ATCC, Istituto Superiore di Sanità, Italy) as antigens: B_1 = Connecticut 5; B_2 = Ohio 1; B_3 = Nancy; B_4 = JVB, Benschoten; B_5 = Faulkner; B_6 = Schmidtt. Anti-mumps virus antibodies were determined with the complement-deviation test (antigens S and V) and anti-cytomegalovirus antibodies were determined by immunofluorescence. Values fourfold or more higher than the basal titer in the 1st mo were regarded as positive.

ICAs. Standard indirect immunofluorescence techiques with fluorescein isothiocyanate were used with human pancreas from a blood group O kidney donor. Undiluted and 1:10–1:40 saline-diluted human serum was used according to Bottazzo et al. (13) to measure both ICAs (IgG) and CF-ICAs. Two operators independently performed the same double-blind measurements with a 0 to ++ scale that had been determined and checked in Bottazzo's laboratory through the exchange of double-blind specimens. The sera that tested positive in up to 1:40 dilution were considered strongly positive (++).

HLA typing. The NIH standard lymphocytotoxicity technique was used to type HLA-A, -B, and -C. All HLA specificities recognized by the WHO nomenclature committee (14) were typed. HLA-DR typing was performed with the B-lymphocyte–toxicity technique used during international workshops (15). DR1, DR2, DR3, DR4, DR5, DRw6, and DRw8 antigens were typed.

Statistical analysis. Fisher's exact test was used to compare HLA frequency in the IDDM patients and controls. *P* values were corrected for the number of comparisons made for each locus. Relative risk (RR) of diabetes was determined by Wolf's formula (16), and odd risk (OR) was determined according to Svejgaard (16). The etiologic and protective fractions were also determined (17). Fisher's exact test and Student's *t* test were used for the other analyses. All averaged values are expressed as means ± SE or SD.

TABLE 2
Percentage positivity of islet cell antibodies (ICA) and complement fixing ICA (CF-ICA) in two concentrations of saline-diluted human serum at onset and quarterly during 12-mo follow-up in insulin-dependent diabetics and sibling and healthy controls

	Insulin-dependent diabetics					Sibling controls (*n* = 52)	Healthy controls (*n* = 126)
	At onset (*n* = 74)	3rd mo (*n* = 68)	6th mo (*n* = 65)	9th mo (*n* = 65)	12th mo (*n* = 64)		
ICA							
1:10	58	48	42	32	30	0	0
1:40	30	28	20	18	20		
CF-ICA							
1:10	39	31	25	22	17	0	0
1:40	17	22	17	17	12		

Mean age (±SE) is 14 ± 1.4 for ICA-positive patients and 26.7 ± 2.7 for ICA-negative patients ($P < .01$).

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TABLE 3
HLA antigen frequency in 74 subjects with insulin-dependent diabetes mellitus (IDDM) and 526 healthy controls (HC)

HLA antigen	Percent positive		P	RR	RR confidence limits	Etiologic fraction	Protective fraction
	IDDM	HC					
Cw5	23.0	11.6	NS				
Cw7	37.8	30.1	NS				
B8	23.0	9.1	.02	2.97	1.50–5.50	0.15	
B18	29.4	14.4	.03	2.51	1.44–4.40	0.18	
Bw39	12.2	3.0	.04	4.46	1.86–10.7	0.10	
Bw62	6.8	4.5	NS				
Bw63	4.1	4.5	NS				
Bw4	51.4	74.9	.001	0.35	0.20–0.58		0.49
Bw6	94.6	76.4	.002	5.32	1.90–14.9	0.77	
DR2	9.7	19.4	NS	0.45	0.20–1.01		
DR3	52.8	17.1	$<10^7$	5.41	3.23–9.06	0.43	
DR4	43.1	18.5	.0000576	3.34	1.99–5.59	0.30	
DR5	13.9	48.2	$<10^6$	0.17	0.09–0.35		0.40

RR, relative risk. For statistical analyses, see MATERIALS AND METHODS. HLA-DR were not typed in 2 IDDM subjects.

RESULTS

Seventy-four cases of acute-onset IDDM were collected from February 1981 through January 1982, corresponding to 5.0/100,000 inhabitants (11.6/100,000 <20 yr, 31 cases). Clinical onset included ketoacidotic coma in 2 cases (2.7%). Polydipsia-polyuria was present in all cases, loss of weight in 88%, asthenia in 80%, and nausea plus vomiting in 25%.

Few patients <5 and >40 yr (both 8%) were observed. The distribution by month of onset of all patients and of the 14 subjects (19%) with an at least fourfold increase in anti-virus antibody titer is shown in Table 1. Five of the 52 healthy sibs (10%) also had a fourfold increase.

The frequency of ICA and CF-ICA at different months after onset of diabetes is shown in Table 2. In the controls, no ICAs or CF-ICAs were detected. Comparison between the ICA-positive patients at onset with those negative throughout the follow-up period showed that the mean age of ICA-positive individuals was significantly lower, but the number of patients positive for anti-virus antibodies was not significantly different in the two groups.

HLA-A, -B, and -C were typed in all 74 patients (Table 3). DR antigens could not be typed in 2 subjects for technical reasons. DR3 and DR4 were closely correlated with IDDM. DR2 was negatively associated only in women (1/36 female patients, 51/314 controls) ($\chi^2 = 4.629$, $P < .05$, RR = 0.34). In men there was no association between DR2 and IDDM (6/36 male patients, 50/212 male controls), but 4 out of 6 DR2 male patients were either DR3 or DR4 positive.

Table 4 shows the HLA phenotypes in which DR3 and/or DR4 were present. The DR3/DR4 phenotype showed a much higher RR (and OR) than those in which only one of these antigens was present. However, an increased RR was also observed when each allele was evaluated separately. Frequency of DR3 in DR4-negative individuals was 61% in IDDM and 20% in controls ($\chi^2 = 34.145$, $P < .0001$); frequency of DR4 in DR3-negative individuals was 53% in IDDM and 22% in controls ($\chi^2 = 19.708$, $P < .0005$).

The HLA-B phenotypes of DR3- and/or DR4-positive patients were analyzed for evidence of preferential involvement of some HLA haplotypes. Contu et al. (18) reported that among DR3 or DR4 patients, some haplotypes are over-represented. In our series, 15 have both DR3 and B8, 18 have DR3-B18, and 5 have DR4-Bw62, which was significantly more frequent than in controls. The relative risks of the three phenotypes are 4.1, 8.4, and 6.5, respectively.

In relating DR phenotype to ICA presence, 3/– and 3/X and 4/– and 4/X were cumulated. There was a significant difference in the frequency of ICA among DR3/DR4 and DRX/X patients ($\chi^2 = 9.15$, Fisher's $Pc = .012$).

No significant correlation was found between DR phenotype and presence of CF-ICA, and ICA persistence was significantly higher ($P < .028$) in DR3/DR4 patients. DR3-

TABLE 4
DR phenotype frequency in 72 subjects with insulin-dependent diabetes mellitus (IDDM) and 526 healthy controls (HC)

DR phenotype	Percent positive		χ^2	RR	RR confidence limits	Odds ratio*
	IDDM	HC				
3/–	16.7	6.1	10.41	3.09	1.51–6.31	8.02
3/X	18.1	10.5	3.63	1.89	0.97–3.66	5.05
3/4	18.1	1.7	47.74	12.66	5.19–3.87	31.17
4/–	8.3	6.1	0.54	1.40	0.57–3.48	3.98
4/X	16.7	11.8	1.39	1.50	0.76–2.94	4.14
X/X	22.2	65.0	48.28	0.15	0.09–0.28	1.00

RR, relative risk; –, no DR antigen determined; X, DR antigen other than DR3 and DR4 serologically determined.
*Calculated with respect to X/X.

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positive individuals were significantly younger than DR3-negative individuals (16.8 ± 5 vs. 24.0 ± 16.9 yr; $P < .05$).

DISCUSSION

The incidence of IDDM in this population is relatively low (3,19,20). Because cases were collected through public hospitals and centers, some might have been lost to other health institutions outside the catchment area. However, the initial care of newly diagnosed diabetics took place only in public hospitals and diabetes centers. There are no private diabetes centers within the study area. When discharged from hospitals, patients are referred to the nearest diabetes center for follow-up. Other studies concerning subjects aged <20 yr show an incidence rate much the same as ours (21). In disagreement with studies on other populations (22,23), clinical onset had no seasonal pattern, although there were statistically insignificant peaks in January and February (19 cases, 26%) and September and October (15 cases, 20%), and a fall in July and August (7 cases, 9%).

Many workers have suggested a viral mechanism for development of IDDM (24,25), but recent reports cast doubt on this view (26–28). In this study, no viral antibodies were observed even when the series was divided into DR3-positive and -negative individuals, although significant changes in antibody titer might have been apparent only in the preclinical stage.

ICA data over time in this series were comparable with those reported by Bottazzo et al. (29–31), i.e., a high percentage of individuals who were ICA positive at clinical onset of diabetes, followed by a gradual decline. Even so, ~42% of the patients were negative both at onset and throughout the follow-up. Clinical onset of diabetes may occur months after the commencement of pancreatic damage, and ICA response may play merely an accessory role as recently reported (32). The only significant difference between the ICA-positive and -negative IDDM cases was in the age at onset, 14.2 ± 1.4 vs. 26.7 ± 2.7 yr ($P < .001$).

DR3/DR4 heterozygotes showed a significantly higher prevalence of ICA-positives when compared with DRX/X phenotype. This point is controversial (33–38). Apparently DR3 is more closely related to IDDM than is DR4 as already reported (39,40), which is different from published observations concerning northern European populations, where DR4 is more closely associated with IDDM than is DR3 (41). This phenomenon can be ascribed to a marked difference in DR gene frequency among Italians compared with the other European populations (42).

The statistical significance of the association between diabetes and DR antigens is much higher than with antigens of other HLA loci (e.g., B8, B18, Cw5), and this can be attributed to linkage disequilibrium between B8 and DR3. The observed association of DR3 and DR4 with some HLA-B antigens suggests that some HLA-haplotypes are more frequent than others in IDDM patients. In this study, B8-DR3, B18-DR3, and Bw62-DR4 are significantly associated with IDDM. This means that neither DR3 nor DR4, when not associated with B8, B18, or Bw62, are a risk factor for IDDM, which is in agreement with the work of other researchers (18,43).

The assumption that DR alleles are in linkage disequilibrium with genes directly involved in the etiology of diabetes or in conferring protection against it leads to the conclusion that, in our series, the linkage disequilibrium of a hypothetical protective gene occurs not only with DR2 but also, and to a greater extent, with DR5. For DR2, most of the male DR-positive patients of this series may also possess a "risk allele" (either DR3 or DR4), thus neutralizing the protective effect of DR2. This is in agreement with a genetic heterogeneity of IDDM. Prospective studies of IDDM patients without exclusion (e.g., patients >20 yr of age) or selection (e.g., familial IDDM) have confirmed heterogeneity in the frequency of HLA-DR phenotypes, as discussed by Nerup et al. (44).

In conclusion, the results of this study suggest that in type I diabetes *1*) viral etiology is not evident, *2*) ICAs offer only a partial pathogenetic explanation, and *3*) genetic and immunologic heterogeneity is evident.

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