Prospective Study of Insulin-dependent Diabetes Mellitus

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SUMMARY

Laboratory study of 109 insulin-dependent diabetics younger than 17 yr of age and resident in greater Montreal at the time of onset of symptoms is reported. The cases were diagnosed during a 2-yr period (1976–1978). Sibling controls were obtained for 72 of the cases studied. Viral titers to Coxsackie B, rubella, and mumps viruses for the 72 patient-sibling pairs showed no difference in geometric mean titers or in change of titer between samples taken at the time of diagnosis and those taken 28 days later. The incidence of positive islet cell antibody in the IDDM cases was 68.0% at the time of diagnosis compared with 56.9% 4 wk later. The comparative figures for sibling controls were 4.2% and 1.4%, respectively. The frequency of HLA B8, B15, B18, and B7 antigens were compared both with the sibling controls and a normal control population. Pairings of high risk HLA antigens were found more frequently in cases than controls. There was no difference in geometric mean viral titers in cases with high risk haplotypes compared with those cases in which such haplotypes were absent.

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The epidemiologic characteristics of insulin-dependent diabetes (IDD) reported from various centers support the association of genetic and environmental factors in the pathogenesis of this condition.[1–3] Seasonal variation in the onset of the disease in conjunction with animal studies in which diabetic syndromes have been produced by viral agents[4] suggest that viral infections may be one of the environmental agents. Recently, the isolation of a strain of Coxsackie virus B4 has been reported from the pancreas of a 10-yr-old male who died during the initial episode of diabetic ketoacidosis. Furthermore, this isolate produced diabetes in susceptible experimental animals and more closely resembled an experimental "diabetogenic strain" than the standard human prototype strain.[5] A specific genetic predisposition to IDD that is closely linked to genes at the major histocompatibility locus (HLA) has been defined.[6,7] Islet cell antibodies (ICA) are frequently demonstrated at the onset of the disease but their significance remains to be clarified.[8]

There are three questions to be asked: (1) is the individual genetically predisposed to develop diabetes more susceptible to Coxsackie virus, (2) how often can a Coxsackie or other viral infection be shown to antedate the development of clinical diabetes in children, and (3) are these related to the development of ICA?

Although comparisons have been made between viral antibody titers and HLA B locus antigens,[9,10] there have been no studies in which viral titers, ICA, and HLA typing were done in a population for which epidemiologic characteristics have been established. Because there are ethnic and socioeconomic differences in the incidence of the disease, it is important that all cases in a given geographic area during a defined period of time and within a stated age group be included to avoid referred or seasonal bias. We have previously reported a register of all new insulin-dependent diabetics less than 17-yr of age, resident in Metropolitan Montreal, in whom the onset of symptoms occurred between January 1, 1971 and December 31, 1977.[11] In the present study, we report a prospective study involving all new cases of IDD occurring in this area between August 15, 1976 and August 14, 1978. Wherever possible, a sibling control living at home was also studied. Originally, a playmate control had been planned but efforts to include such children in the study proved to be impossible because of lack of motivation and compliance. We examined viral antibody titers, antigens of the A and B loci of the HLA complex, and frequency and titers of islet cell antibodies.

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MATERIALS AND METHODS

During the 2 yr under study, 131 patients, all of Caucasian origin, met the following criteria: (1) less than 17 yr of age at the time of onset of symptoms, (2) resident of Greater Montreal at the time of diagnosis, and (3) insulin-dependent. One hundred and nine of these 131 patients consented to take part in the study, and sibling controls were obtained for 72 of the consenting group. Where there was more than one sibling in the family, the sibling nearest in age to the patient was taken.[1] Serum samples were taken from each patient and the sibling control at the time of diagnosis and 1 mo later. During the first year, all samples were tested for viral antibody titers to Coxsackie viruses B1-6, rubella virus, and mumps virus. During the second year, they were tested only for Coxsackie viruses B1-6. The duration between the date of historical onset of diabetic symptoms and the date of diagnosis (and therefore the collection of the first serum sample) varied between 2 days and 151 days, with 72% being 31 days or less.

Laboratory methods. Antibody titers to Coxsackie B1-6 were performed using the replicate neutralization test.[12] The initial dilution was 1:10 and the titers were calculated using the formula of Reed and Muench.[13] The strains of virus used were those supplied as WHO Reference Strains, namely, Cox. B1-Conn. 5: Cox. B2-Ohio 1; Cox. B3-Nancy; Cox B4-JVD; Cox, B5-Faulkner; and Cox. B6-Schmitt. Rubella titers were performed using a hemagglutination inhibition method beginning with a dilution of one quarter.[14] Mumps titers were performed using a complement fixation test using the S and V antigens.[15]

Islet cell antibody activity was detected using standard indirect immunofluorescent techniques.[16] Human pancreas was obtained from blood group O cadaveric renal transplant donors, and 5-μm cryostat sections were air dried and fixed in acetone at room temperature for 10 min. Initial screening for islet cell antibody was carried out with undiluted human sera and final studies were carried out starting with a 1:2 serum dilution. Fluorescein conjugated monovalent goat anti-human IgG, IgA, and IgM sera (Kent Laboratories) were employed at a dilution of 1:10.

All islet cell antibody detected was of the IgG class and demonstrated the characteristic pattern of cytoplasmic fluorescence in all pancreatic islet cells. Final determinations of islet cell antibody titer were carried out on a single donor pancreas, which was stored at −70°C for no longer than 8 wk before use. All samples were read in a double-blind fashion.

HLA antigens at the A, B, C, and DR loci were determined by a standard lymphocytotoxicity technique using UCLA trays.[17] Twenty specificities were tested at the A locus, 31 at the B locus, and 8 at the C loci.

TABLE 1
Epidemiologic characteristics of insulin-dependent diabetes in Montreal during the period from August 1976 to August 1978

	Year 1		Year 2		Total	
Number of cases	69		62		131	
Incidence per 100,000	8.1		7.3		7.7	
Male (%)	41.4		53.2		46.6	
Females (%)	58.6		46.8		53.4	
Mean age	9.6		8.9		9.2	
	No.	(%)	No.	(%)	No.	(%)
Onset of symptoms						
Jan.	5	7.3	5	8.1	10	7.6
Feb.	6	8.7	3	4.8	9	6.8
Mar.	6	8.7	3	4.8	9	6.8
April	10	14.5	7	11.3	17	12.9
May	3	4.4	6	9.7	9	6.8
June	5	7.3	1	1.6	6	4.6
July	3	4.4	4	6.4	7	5.3
Aug.	3	4.4	12	19.3	15	11.4
Sept.	5	7.3	5	8.1	10	7.6
Oct.	3	4.4	4	6.4	7	5.3
Nov.	11	15.9	9	14.5	20	15.2
Dec.	9	13.0	3	4.8	12	9.1

TABLE 2
Geometric mean titers (GMT) and numbers of fourfold or greater rises to Coxsackie B1-6, rubella, and mumps viruses among insulin-dependent diabetics (IDD) and their sibling controls

	Case control pairs						All cases (109 IDD)		
	72 IDD			72 Siblings					
	GMT			GMT			GMT		
Virus	0 Day	28 Day	No. of fourfold rises	0 Day	28 Day	No. of fourfold rises	0 Day	28 Day	No. of fourfold rises
Cox. B1	27	40	3	24	36	2	26	31	4
B2	52	64	0	44	60	1	48	56	4
B3	60	57	6	60	60	0	50	52	13
B4	72	109	4	83	60	2	61	89	6
B5	39	39	2	25	23	2	32	32	3
B6	5	5	0	5	5	0	5	6	0
	43 IDD			43 Siblings			61 Cases		
Rubella	35	37	2	50	48	1	28	33	5
Mumps S	5	4	0	5	5	0	5	5	0
V	9	10	1	10	8	0	9	10	1

* Only samples from the first year of the study were tested against rubella and mumps viruses.

Statistical analysis. To make the most effective comparison between cases and controls, taking into account both viral titers at 0 and 28 days and the matching of pairs, Hotelling's matched T^2 tests were carried out.[18] In addition, geometric mean titers (GMT) on both 0 and 28 days from those cases without sibling controls were compared descriptively with the cases that had controls. Viral antibody titers were correlated with the presence or absence of ICA and with three B antigens whose presence had previously been reported to correlate with an increased risk of IDD (B8, B18, and B15) in Caucasian populations similar to ours. Furthermore, all of the laboratory measures were compared with the month of onset in an attempt to demonstrate seasonal clustering.

RESULTS

Epidemiology and family history. Epidemiologic data for the 131 patients diagnosed in Metropolitan Montreal during the 2 yr of the study are summarized in Table 1. There was clustering of cases in April and November. One sibling control developed IDD within 2 wk of his sister. In another family, a noncontrol sibling became diabetic within 6 mo of diagnosis of the proband. There were eight previously diagnosed diabetic siblings among 206 siblings at risk (3.9%).

Viral titers. The data for the 72 patient-sibling pairs revealed no difference in the mean geometric titers for Coxsackie B1-6, rubella, or mumps antibodies (Table 2). Neither was there a significant difference in the change in mean titers between the day 1 and day 28 samples. In addition, we looked for fourfold or greater rises in titers in all patients (N = 109) and in the sibling controls (N = 72) (Table 2). In all, 30 of 109 patients had a fourfold rise in titer to one or more Coxsackie B virus antigen as compared with 7 of 72 controls. The details of the 29 patients exhibiting these fourfold rises are detailed in Table 3. The largest number, 13, had antibody responses to Coxsackie B3. Twelve of these occurred in the first year of the study, but there was no seasonal clustering. The initial mean titer of antibody B3 in the 13 children was 12. This was lower than the initial mean titer among the group as a whole, which was 60. Patients 13 and 21 are siblings who became diabetic within 6 mo of each other. Patient 10 has a diabetic sibling who was diagnosed during the study, but he had low antibody titers to all Coxsackie viruses.

Islet cell antibodies (Table 4). The incidence of positive islet cell antibody at the time of diagnosis in the insulin-dependent diabetics was 68% and fell to 56.9% 4 wk later. In sibling controls, 3 of 72 (4.2%) had detectable islet cell antibody at the time of diagnosis, which fell to 1.4% 28 days later. These figures can be compared with an incidence of positive islet cell antibodies of 0.95% (2 of 211) in a normal population from the same laboratory. The highest islet cell antibody titers in the sibling controls was 1:4, whereas the patients had titers as high as 1:128. In the 13 cases with a fourfold or greater rise in titer to Coxsackie B3, the mean ICA titers rose from 9.2 at the time of diagnosis to 17.1 at 28

TABLE 3
Details of the 31 patients exhibiting fourfold rises in Coxsackie virus

			Onset month		Interval between Sym and Dx (days)	Coxsackie virus type antibody titer										ICAB Titer		HLA	
						B1		B2		B3		B4		B5					
Pt.	Age	Sex	Sym	Dx		0	28	0	28	0	28	0	28	0	28	0	28	A	B
1	9	F	1	2	23	226	1280									16	32	2,—	15,W44
2	11	F	4	5	20	5	20									5	16	2,—	5,15
3	10	M	12	12	3	5	28									128	16	2,—	27,40
4	16	F	1	2	21	5	20									16	32	2,3	18,40
5	7	F	11	12	4			40	160							16	8	2,3	7,W39
6	1	F	7	7	21			5	40							—	—	2,—	14,W44
7	2	M	11	12	37			5	20							16	0	2,—	5,18
8	8	F	4	6	60			20	80							0	0	1,29	8,—
9	12	F	9	12	60					5	80					4	4	24,28	28,40
10	12	M	7	8	21					20	80					2	8	3,12	9,18
11	15	F	1	4	73					5	40			57	320	8	32	1,2	15,—
12	10	M	3	4	31					10	113					0	0	2,—	15,18
13	12	M	5	5	7					28	113					0	0	3,29	7,W21
14	11	M	9	9	2					20	80					0	0	2,W26	W38,W35
15	15	M	11	11	10			20	80	14	80	5	20			4	4	3,30	8,39
16	15	M	10	11	13					5	80					32	32	W23,W32	W21,W35
17	9	F	11	12	30					20	160					64	128	24,28	W42,W35
18	7	F	12	1	42					10	160					0	8	1,2	8,18
19	13	F	3	4	31					5	40					4	4	28,32	15,—
20	6	M	6	6	21					14	57					2	2	3,—	7,17
21	10	F	10	10	7					5	40					0	0	3,29	7,W21
22	13	F	12	1	16							40	160			0	0	1,2	8,12
23	10	F	11	11	14			5	40			227	1000	5	57	128	128	2,28	15,18
24	13	F	12	12	9							320	1000			0	0	10,29	16,22
25	13	F	7	8	31							5	28			4	16	3,28	W35,W38
26	14	M	9	9	21							57	226			—	—	2,28	15,W51
27	3	M	7	7	3							5	640			16	—	2,32	W44,W51
28	11	F	1	5	90									5	28	0	2	24,30	13,40
29	9	M	11	11	14									40	113	0	0	1,2	7,—
30	15	M	11	12	21									5	57	0	0	23,24	18,—

TABLE 4
Islet cell antibody determinations in insulin-dependent diabetes (IDD) and sibling controls

	Case control pairs						
	IDD (72)		Siblings (72)		All IDD* (105)		
	0 Day	28 Days	0 Day	28 Days	0 Day	28 Days	Population control (211)
Serum dilution 1:2							
No. positive	49	41	3	1	71	62	2
% positive	68.0	56.9	4.2	1.4	74.8	65.2	0.9
Mean titer	16.6	13.1	<1.0	<1.0	15.8	12.1	<1.0

Number of children studied per group is shown in parentheses.
* Four cases were not tested for ICA.

days. This was in contrast to the decrease in mean titer from 19.2 to 11.0 in all other cases. There were 23 IDD in whom islet cell antibody was never detected; five had HLA antigen B8, two had B15, and seven had B18. Fifteen were female and ten were male.

HLA typing (Table 5). The frequency of HLA B antigens 8, 15, 18, and 7 were compared with 263 Caucasian Montreal primiparous women whose ethnic distribution was similar to that of our patients. These antigens were chosen for comparison because they have previously been shown to confer either increased (B8, 15, 18) or decreased (B7) risk to IDD in other populations. In the present study, pairing of antigens (B8, B15, or B15, B18) was found to occur more frequently in the cases than in their sibling control or in the control populations. Five of the 107 cases had either B18/B15 or B8/B15 as compared with only one sibling control. The sibling who became diabetic 6 mo after her brother was HLA identical with the proband but neither had any of the "high risk" B antigens. Seven of the eight previously diagnosed IDD siblings were typed; six shared both haplotypes with the case; one shared one haplotype. Two pairs were B18/B15 heterozygotes. There was no difference in the geometric mean viral titers of those cases with the high risk haplotypes as compared with those in which these haplotypes were not present. There was no seasonal clustering of onset of symptoms in patients with high risk haplotypes. Twelve sibling controls were HLA identical to the patients but none had positive ICA titers of 1:2 or greater. The HLA identical siblings did not show a fourfold rise in antibody titers to any of the Coxsackie viruses. Case 17 had a diabetic sibling diagnosed many years previously. Of the 13 patients with responses to Coxsackie B3, 5 had HLA antigens B8, B18, or B15. One of these five was B15/B18 heterozygous, one was B8/B18 heterozygous, and two others had a single B15 demonstrated, although parental typing has not been done to determine if they are, in fact, homozygous for B15.

DISCUSSION

Current theories of the pathogenesis of insulin-dependent diabetes mellitus posit a genetic susceptibility which is associated with certain HLA types as the B,D and DR loci interacting with an environmental insult to produce selective damage to the beta-cells of the pancreas.[6,20] Diabetes has been produced in mice with viral infections.[21–23] Three viruses, Coxsackie B, mumps, and rubella, have been suggested as inciting agents in human diabetes.

It was the observation of a seasonal variation in the month of diagnosis of IDD which led Gamble and his colleagues to suggest that the Coxsackie B group of viruses might be involved in the initiation of the pancreatic pathology.[24] These investigators later studied 110 cases of newly diagnosed IDD less than 30 yr of age in whom blood samples were drawn 0–18 days (mean 5.5 days) following diagnosis.[9] They found a significant increase in Coxsackie B1 antibody titer in subjects positive for HLA antigens B8 and B15 compared with subjects not having these antigens. They also found a higher percentage (73% as compared with 30%) of positive antibody in patients presenting in winter months (January through March) than in autumn months (September through November). Dippe studied a large isolated popula-

TABLE 5
Frequency of HLA B antigens

	Pairs										
	Cases (72)		Controls (72)		Total cases (109)						
	No.	%	No.	%	No.	%	Population control (263)		X^2†	P value	Rel. risk
HLA B 8	16	22.5	15	20.8	31	28.4	31	11.8	14.2	<0.0001	2.41
HLA B 15	12	16.9	12	16.6	19	17.4	20	7.6	6.9	<0.008	2.29
HLA B 18	19	26.8	14	15.9	32	29.3	31	11.8	15.2	<0.0001	2.48
HLA B 7	10	14.1	8	11.6	13	11.9	50	19.0	2.27	<0.131	0.57
HLA B8/B15	1	1.4	0	0	2	1.9	1	0.38			
HLA B18/B15	4	5.6	1*	1.4	6	5.6	1	0.38			
	5	6.9	1	1.4	8	7.3	2	0.76	10.36	<0.001	7.24

* Became diabetic.
† Comparison between total cases and population control.
Relative risk was calculated according to the method of Svejgaard (1974).[19]

tion which has sustained an epidemic of Coxsackie B4 and found increased incidence of IDD.[25] The significance of this report has been questioned in view of the fact that no information on the HLA typing of these individuals is available. Other small groups of diabetics not showing evidence of recent Coxsackie infection have been reported.[26] However, reports of isolated cases of diabetes following Coxsackie virus infection have appeared.[27] In one, a virus of an atypical Coxsackie B strain was isolated from the pancreas.[4]

We found no difference in mean viral titers between cases and controls in any of the Coxsackie B viruses at either day 0 or day 28 after diagnosis nor was there any difference in the change in antibody titers between cases and controls. However, 13 patients did have a fourfold rise in titers to Coxsackie virus B3, and 16 to one of the other Coxsackie B virus. If we compare the number of patients (N = 15) and controls (N = 7) exhibiting fourfold rises among the pairs, the difference is not statistically significant ($X^2 = 1.4$, $P < 0.05$). Unfortunately, about half of the fourfold rises in antibody titers occurred in patients without sibling controls. We recognize that a schoolmate or neighborhood child would have been a desirable additional control but, as stated earlier, our efforts in this direction were unsuccessful. These data, therefore, cannot answer the question as to whether the child genetically predisposed to diabetes is more likely to develop Coxsackie infection than a child from a control population. They can only suggest that there is a subgroup of diabetic patients who had serologic evidence of a recent Coxsackie infection at the time of diagnosis. They further indicate that these cases occurred randomly throughout the year and paralleled the distribution of all the cases. The time interval between onset and symptoms and the time of diagnosis was not different in this subgroup. Seventy-one percent of the overall sample had symptoms for less than 1 mo, and 79% of the subgroup with fourfold rises had symptoms for less than 1 mo. The age range in the fourfold subgroup was from 1 yr to 16 yr (mean 9.4 yr) and had the same distribution as that of the entire group in whom the mean age of onset was 9.2 yr.

Mumps-virus infection has been implicated on the basis of anecdotal accounts of diabetes following parotitis by days or months[20] and by the study of Sultz et al., who noted an increased incidence several years after a mumps epidemic.[28] Rubella virus has been suggested following the description of Meuser et al.[10] of diabetes mellitus in children with the congenital rubella syndrome. The affected individuals have a high frequency of HLA B8 antigen[10] and of DR3 and DR4.[29] In the present study, as in others,[9] there was no serologic evidence of recent rubella or mumps infection. Two of the patients had had recent rubella immunizations; one exhibited a fourfold rise in titer between day 0 and day 28. The other did not. We were unable to control for the effects of prior immunizations on rubella and mumps titers; for most of the patients, immunization was remote in time and records were poorly kept. This is not surprising, as the latent period suggested by the above cited studies has been measured in years or decades.

The data confirm the genetic predisposition associated with specific B antigens of the HLA locus. There was a striking increased relative risk for individuals having the pairs of B antigens B8/B15 and B18/B15. Recent studies indicate that susceptibility to insulin-dependent diabetes is more closely associated with antigens coded at the D and DR loci that are closely linked with the B antigens. In some populations, B8 and B18 are in strong linkage disequilibrium with DR3 and B15 and DR4. DR typing of some of the children in this population in conjunction with a study of familial diabetes[30] indicates that more than 90% of our children, whose age of onset of symptoms is 17 yr or less, have either DR3, DR4, or DR3/DR4. In our population, B8 and B18 are often associated with DR3. Although B15 is associated with DR4, a number of other B antigens (B40, BW44, BW45) are also found in combination with the DR4 specificity. It is, therefore, not surprising that we could demonstrate no relation between any HLA B antigen and season of onset or viral antibody titer, and it will be important to examine the data when correlation with the two major DR susceptabilities can be examined.

More than two-thirds of the diabetics had ICA in titers of 1:2 or greater at the time of diagnosis. This is in agreement with other recent reports.[31] Only a small fraction of the controls had ICA; when present, the titers were low. In general, ICA titers decreased during the first month following diagnosis. The presence of IgG ICA suggests that the release of islet antigen and hence of initial islet damage must have antedated the date of diagnosis by at least 3–4 wk. In this regard, it is of interest that the mean ICA titer in the subgroup of cases with evidence of recent serologic conversion to Coxsackie virus rose between 0 and 28 days (from 9.2 to 17.1), whereas the mean titers fell (from 19.2 to 11.0) in the rest of cases. However, as is indicated in Table 3, there is considerable variation in titers and the rise is accounted for by a relatively few patients.

Finally, specific mention should be made of the case-sibling control pair in which the sibling became diabetic 2 wk after diagnosis of the case. The case (Patient 1, Table 3) exhibited a fourfold increase in viral antibody to Coxsackie virus B4 and also possessed moderate antibody titers to B3. In addition, she had high ICA titers in both 0 and 28 day samples. The male sibling living in the same household possessed very low viral antibody levels and ICA were never detected in successive three monthly samplings. The children are HLA identical possessing the A2,B15,DR4/A28,B18,DR3 genotype. Both children are clearly insulin-dependent and 3 yr later require 0.8 and 1.2 U/kg of insulin daily.

We conclude that there is a strong genetic predisposition to the development of the childhood form of insulin-dependent diabetes. While some seasonality was observed, the viral data do not support the suggestion that recent viral infection was associated with the onset of clinical IDD. Seventy-four percent of the patients did not have evidence of rising viral titers in association with the early clinical manifestation of the disease. Those who did exhibit fourfold rise in titer did not show a seasonal trend. Thus it is not possible to relate the seasonal variation observed to recent viral infection. Serologic evidence of remote viral infections may, of course, no longer be present at the time of diagnosis. When present, ICA are a marker of pancreatic disease. At the present level of sensitivity of this measurement, not all children with the disease have detectable levels of ICA antibodies at the time of diagnosis. It seems likely that a number of different environmental factors may initiate pancreatic pathology. Repeated insults may occur before final decom-

pensation occurs. The genetic contribution may be related to the inability of the islet cell mass to withstand this attrition because of a congenitally deficient islet cell mass, because of programmed inability to regenerate beta-cells, or because of the initiation of an autodestructive process.

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