HLA antigens and other risk factors in the development of retinopathy in type 1 diabetes

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SUMMARY Factors possibly influencing the development of diabetic retinopathy were studied in 112 randomly selected type 1 diabetics having no or minimal retinopathy (group A) and in 82 type 1 diabetics with known severe diabetic retinopathy. The latter comprised those with severe background retinopathy (group B, $n=17$) and those having proliferative retinopathy without (group C, $n=38$) and with (group D, $n=27$) diabetic nephropathy. Nonretinopaths (group A) were of similar sex ratio, body weight, and age at diagnosis of diabetes but had been diabetic longer ($p<0.001$) and were thus older ($p<0.001$) than retinopaths (groups B-D). The distribution of HLA antigens of the A, B, and C loci was similar in nonretinopaths and retinopaths with the exception that HLA B7 showed a reduced ($p<0.05$) prevalence in the retinopaths (6% versus 17%) and was singularly underrepresented in group D, where no patients had this antigen. Mean postprandial plasma glucose and HbA_{1c} concentrations were higher ($p<0.01$ and $p<0.001$) and cigarette smoking was more prevalent ($p<0.01$) in the retinopathy groups B-D than in group A. Systolic and diastolic blood pressures were similar in groups A-C, with higher ($p<0.001$) values only in group D. There was no association between insulin antibody binding in the serum or measurable plasma C-peptide immunoreactivity and retinopathy status. The risk of development of diabetic retinopathy in type 1 diabetes may be related to HLA-associated genetic factors and to cigarette smoking.

In order to evaluate the possible factors which may influence the incidence, severity and progress of retinopathy in type 1 diabetics we have examined the frequencies of the HLA antigens of the A, B, and C loci, selecting only those diabetics who had either developed severe diabetic retinopathy or exhibited an apparent immunity to the development of other than very mild background diabetic retinopathy. Since duration of diabetes is possibly the most important determinant of the development of diabetic retinopathy, we have taken particular care to select retinopaths of short duration of diabetes and nonretinopaths of long duration of diabetes. In addition to considering HLA antigen frequencies we have compared the 2 groups of diabetics in respect of

postprandial plasma glucose concentration, total glycosylated haemoglobin (HbA_{1c}), insulin antibody binding by serum, prevalence of diastolic and systolic hypertension, and smoking history. Finally, we have shown that all subjects studied were type 1 diabetics by confirming an absent or only just measurable postprandial C-peptide concentration in the plasma. We thus avoided the risk of inadvertently including subjects with 'maturity onset diabetes of the young' in the group of nonretinopaths.

Patients and methods

PATIENTS

All 194 diabetics studied attended the Diabetic and Dietetic Department, Royal Infirmary, Edinburgh, having presented with clinical features of type 1

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diabetes before the age of 35 years and having been treated with insulin from the outset.

Nonretinopaths. One hundred and twelve unrelated diabetics (49 males, 63 females) were randomly selected from a cohort of patients who are the subject of a long-term prospective study of the development of retinopathy. All such patients were known to have been diabetic for at least 15 years, in which time they had no ophthalmoscopic evidence of diabetic retinopathy.

Retinopaths. Seventy-three unrelated diabetics (44 males, 29 females) with severe background or proliferative retinopathy were studied representing all available type 1 diabetics of duration of diabetes less than 25 years who had been referred to the Princess Alexandra Eye Pavilion, Edinburgh, during 1972–8 for treatment of diabetic retinopathy, from the Diabetic and Dietetic Department, Royal Infirmary, Edinburgh. In order to correct for the male preponderance found in this group of patients a further 9 female retinopaths were included whose proliferative retinopathy was diagnosed in 1979–80.

Nondiabetic controls. One hundred blood donors (50 males, 50 females) were HLA typed.

METHODS

All diabetics' fundi were examined ophthalmoscopically through dilated pupils by one observer (B.F.C.), who assessed the corrected near visual acuity (by standard reading test types) in each patient together with blood pressure in the sitting position after resting quietly for at least 5 min. Patients receiving antihypertensive treatment whose diastolic and/or systolic pressures had previously been recorded as greater than 85 and 155 mmHg, respectively, were identified.

Past and present degrees of proteinuria were assessed by Multistix (Ames Co.).

A questionnaire regarding cigarette smoking habits and family history of diabetes was completed by each diabetic.

A single venous blood sample was withdrawn from each diabetic 1–2 hours after breakfast and was analysed in the following manner. HLA typing was carried out in 104 (93%) nonretinopaths and in all retinopaths (n=82) and nondiabetic controls (n=100). Diabetics and controls were typed concurrently by the standard National Institutes of Health (NIH) lymphocyte cytotoxicity technique against a panel of 120 antisera defining the following locus A, B, and C specificities: HLA A 1, 2, 28, 3, 9 (23, 24), 10 (25, 26), 11, 19, 29, 30, 31, 32, 33, HLA B 5, 7, 8, 12, (44, 45), 13, 14, 15, 16, (38, 39), 17, 18, 21, (49, 50), 22, 27, 35, 37, 40, BW4 and BW6. HLA C CW3 CW4.

In all (n=112) nonretinopaths and 79 (96%)

retinopaths the following investigations were carried out. HbA_{1c} was measured by a modification¹ of the column chromatographic technique of Kynoch and Lehmann.² Reversible and stable forms of HbA_{1c} were not separated by dialysis of red cells.³ Between-batch precision, estimated by repeat analysis of specimens from patients, showed a coefficient of variation (CV) of 3.5%. The normal range in non-diabetics in our laboratory is 5.2–8.4%.

Plasma glucose was estimated by a standard glucose oxidase method.⁴

The binding of ¹²⁵I-labelled beef insulin by serum samples was determined by a method using polyethylene glycol separation.⁵ The nonspecific binding did not exceed 1.5%, and all samples were analysed in one assay, with precision of 2.5% (CV) across the range. Results are expressed as percentage of labelled insulin bound.

C-peptide was measured by radioimmunoassay by a modification of the method described by Heding.⁶ Antibody M1230 raised against human C-peptide was used at an initial dilution of 1/25 000. The detection limit of the assay was 0.04 nmol/l (calculated as 2 standard deviations (SD) from 0). Cross-reaction with human proinsulin is 11% on a molar basis. The interassay coefficient of variation was 6%. Antibody M1230, human C-peptide standard, and iodinated tyrosylated C-peptide were gifts of the Novo Research Institute, Copenhagen, Denmark.

Comparison of groups was by Wilcoxon rank sum, Kruskal-Wallis, or chi-square tests as appropriate, and relationships between variables were tested by Kendall rank correlation (τ).

Results are presented as mean $\pm$ (SD) unless otherwise stated.

Results

Thirty-one nonretinopaths (28%) had minimal diabetic venous changes and/or less than 5 microaneurysms in each fundus, while the remaining 81 (72%) nonretinopaths had no ophthalmoscopic evidence of diabetic retinopathy. All 112 diabetics were assigned to group A and hereafter continue to be referred to collectively as nonretinopaths. Seventeen retinopaths (8 males, 9 females; group B) had severe background retinopathy as evidenced by widespread microaneurysms, blot, flame, or subhyaloid haemorrhages with or without hard or soft exudates but did not exhibit and had not previously exhibited new vessel formation. Sixty-five retinopaths (37 males, 28 females) had proliferative diabetic retinopathy and were divided into patients without (18 males, 20 females; group C) and with (18 males, 9 females; group D) diabetic nephropathy (vide infra).

Table 1 Clinical characterisation of nonretinopath and retinopath

	Nonretinopath	Retinopath		Significance
		Severe background	Proliferative	Nonretinopath vs. retinopath
Number of subjects	112	17	65	—
Sex (% male)	44	47	55	NS
Age (years)	46±14	34±8	37±11	p<0.001
Duration of diabetes at time of study (years)	26±10	12±4	21±7	p<0.001
Duration of diabetes at diagnosis of severe background and proliferative retinopathy (years)	—	12±3	18±6	—
Insulin regimen: daily dose (units)	59±23	60±28	64±30	NS
*Receiving conventional beef insulin (%)	72	65	71	NS
Receiving once daily insulin (%)	56	47	52	NS
†Ideal body weight (%)	100±13	103±14	99±13	NS
Diabetic first-degree relative (%)	21	12	26	NS
Visual acuity: better eye	5.4±1.6	6.3±4.6	8.3±10.6	—
worse eye	7.2±7.0	7.0±5.1	23.1±20.3	—
§Proteinuria grade 0	104	16 (1)	32 (1)	
1	6	0	6	
2	2	0	17 (2)	
3	0	1 (1)	10 (7)	

*Patients not on conventional beef insulin had been converted to highly purified pork insulin up to 3 years prior to time of study.

†Metropolitan Life Assurance Tables, 1959.

§Proteinuria grade: 0=no proteinuria; 1=intermittent proteinuria; 2=persistent proteinuria; 3=nephrotic syndrome—patients with raised creatinine in parenthesis.

Results are expressed as mean±SD.

Owing to the method of patient selection nonretinopath had been diabetic longer ($p<0.001$) and were older ($p<0.001$) than retinopath, but there was no significant difference in sex ratio, daily insulin dose, percentage of ideal body weight, insulin regimen, or family history of diabetes (Table 1) between nonretinopath and retinopath. The visual acuity in each eye of proliferative retinopath was worse ($p<0.001$ and <0.001) than that of nonretinopath (Table 1).

The prevalence of proteinuria and renal failure (serum creatinine $>150 \mu\text{mol/l}$) in nonretinopath and retinopath is also shown in Table 1. Patients with persistent proteinuria or the nephrotic syndrome (without evidence of other than diabetic renal disease) are defined as having diabetic nephropathy.

HLA TYPING

Table 2 shows the distribution of HLA antigens in the subjects studied which are reported to be over- or

Table 2 HLA antigen frequencies (%) in study group

HLA	Diabetic groups						Controls	Significance			
	A	B	C	D	B+C+D	A+B+C+D		(1)	(2)	(3)	(4)
	n= 104	17	38	27	82	186					
A1	48	47	50	56	51	50	31	NS	NS	p<0.01	NS
B7	17	12	8	0	6	12	26	NS	p<0.05	p<0.01	p<0.05
B8	46	47	58	55	55	50	23	NS	NS	p<0.001	NS
B15	21	6	13	22	15	18	10	NS	NS	NS	NS
B18	4	18	8	15	12	7	5	NS	NS	NS	NS
A1+B8	42	29	47	52	45	43	18	NS	NS	p<0.001	NS
B8-A1	4	18	10	4	10	6	5	NS	NS	NS	NS
B8+B15	6	6	5	11	7	6	2	NS	NS	NS	NS

Significance: (1) Among groups B, C, and D. (2) Groups B, C, and D vs. group A. (3) Groups A, B, C, and D vs. controls. (4) Group A vs. Groups C and D.

Group A=nonretinopath. Group B=severe background retinopath. Group C=proliferative retinopath without diabetic nephropathy. Group D=proliferative retinopath with diabetic nephropathy.

Table 3 Risk factors in nonretinopaths and retinopaths

	Diabetic groups				Overall comparison
	A	B	C	D	
Number of subjects	112	17	36	25	
HbA _{1c} (%)	12.1±1.8	15.0±2.5	13.3±2.1	13.6±2.5	p<0.001
Postprandial plasma glucose (mmol/l)	12.6±5.8	18.7±8.2	14.9±8.2	17.1±9.9	p<0.05
Beef insulin antibody binding (%)	23.2±16.1	17.2±13.0	21.7±18.8	16.4±12.4	NS
Plasma C-peptide* (nmol/l)	0.08±0.04 (0.04–0.29)	0.09±0.04 (0.04–0.17)	0.10±0.13 (0.04–0.47)	0.05±0.06 (0.04–0.06)	NS
Diastolic hypertension (%) (≥85 mmHg)	14	0	11	56	p<0.001
Systolic hypertension (%) (≥155 mmHg)	12	6	3	41	p<0.001
Smoking history: smoking at time of study (%)	38	59	54	69	p<0.05
number of cigs/day [†]	20±12	21±8	21±10	24±11	NS
years smoked [‡]	21±10	13±4	18±8	17±7	p<0.05

Results expressed as mean±SD, or range (in parentheses).

*In patients with measurable C-peptide concentration only.

[†]Smokers only.

underrepresented in type 1 diabetics in this or previous studies. HLA A1 and B8 were significantly ($p<0.01$ and <0.001) overrepresented (relative risks=2.18 and 3.34) and B7 significantly ($p<0.001$) underrepresented (relative risk=0.40) in all diabetics (groups A-D) in comparison with controls. The combination of A1 and B8 was more frequently ($p<0.001$) found in all diabetics (groups A-D) than in controls (relative risk=3.51).

The frequency of HLA B7 was greater ($p<0.05$) in nonretinopaths (group A) than in all retinopaths (groups B-D) and greater ($p<0.05$) than in diabetics with proliferative retinopathy (groups C and D). None of the 27 patients in group D exhibited HLA B7. The frequencies of inferred homozygosity of HLA B7 and B8 were 3% and 15% in diabetic nonretinopaths, 0% and 15% in diabetic retinopaths, and 2% and 1% in nondiabetic controls.

HbA_{1c} AND POSTPRANDIAL PLASMA GLUCOSE CONCENTRATIONS

Table 3 shows the mean HbA_{1c} and postprandial plasma glucose values of diabetics in groups A-D. There was no significant difference between plasma glucose values of patients in groups B-D whose overall mean value (16.4±8.8 mmol/l) was significantly higher ($p<0.01$) than that of patients in group A (12.6±5.8 mmol/l). Similarly, the mean HbA_{1c} value of patients in groups B-D (13.8±2.4%) was significantly higher ($p<0.001$) than that (12.1±1.8%) of patients in group A. There was a significant correlation between plasma glucose and HbA_{1c} when retinopaths and nonretinopaths were considered together ($r=0.48$, $p<0.001$) or separately ($r=0.46$, $p<0.001$; $r=0.22$, $p<0.01$). The relationship

between plasma glucose and HbA_{1c} was similar in diabetics with and without a raised serum creatinine concentration. There was no significant relationship between HbA_{1c} or plasma glucose and any HLA antigen when considering retinopaths or nonretinopaths. There was no significant relationship between visual acuity and HbA_{1c} or plasma glucose concentration.

Current smokers had a higher mean HbA_{1c} level than nonsmokers (13.2 versus 12.4%; $p<0.05$), but this is largely accounted for by the higher proportion of smokers among retinopaths. The mean HbA_{1c} of smokers within each of the groups A-D, while significantly higher than that of nonsmokers, is not significantly so in any group.

INSULIN ANTIBODY BINDING

The mean level of insulin antibody binding was not found to differ significantly among patients of groups A-D (Table 3). However, insulin antibody binding correlated significantly with age at study ($r=0.18$, $p<0.001$) and duration of diabetes ($r=0.16$, $p<0.01$). Mean insulin antibody binding in patients taking once daily insulin (25.4±16.8%) was higher ($p<0.001$) than in those taking twice daily insulin (16.7±14%), while patients taking conventional beef insulin had higher ($p<0.001$) insulin antibody binding (23.8±15.9%) than those taking highly purified pork insulin (15.6±15.2%). There was no significant relationship between insulin antibody binding and daily insulin dose or plasma C-peptide concentration. Wilcoxon rank sum tests show mean insulin antibody binding to be lower ($p<0.01$) in HLA B8 positive diabetics (18.2%) than in HLA B8 negative diabetics (25.0%) and to be higher ($p<0.05$) in HLA B15

positive diabetics (27.1%) than in HLA B15 negative diabetics (20.4%). The association between B15 and high insulin antibody binding was independent of the association between B8 and low insulin antibody binding. Mean insulin antibody binding was not significantly different in B7 positive (26.9%) and negative diabetics (20.8%).

C-PEPTIDE

None of the nonretinopathies studied had a plasma C-peptide concentration greater than 0.29 nmol/l. No significant difference was observed in the frequency with which a measurable C-peptide concentration was found in patients of groups A, B, C, and D (30, 47, 25, and 13%). Table 3 shows that the mean plasma C-peptide concentration was not significantly different within groups A-D.

BLOOD PRESSURE

There was no significant difference in the prevalence of systolic or diastolic hypertension (>155 and >85 mmHg) in patients of groups A-C (Table 3). However, the prevalences of systolic and diastolic hypertension in group D (41 and 56%) were significantly greater ($p < 0.001$ and < 0.001) than in groups A-C (10 and 12%).

SMOKING

Table 3 shows the proportions of patients in groups A-D who were smoking cigarettes at the time of study and provides details of daily number of cigarettes smoked and duration of smoking in past and present smokers. Although retinopathies (groups B-D) included more ($p < 0.01$) smokers (60%) than non-retinopathies (38%), this difference was mainly accounted for by the high prevalence of smoking in group D (69%), being significantly greater ($p < 0.05$) than in the other 3 groups of diabetics (44%). These differences were not accounted for by the age differences in patients in groups A-D. There was no significant difference between the prevalence of smoking in patients of groups B and C combined (56%) and that of patients in group A or group D. The significantly longer smoking history in group A is presumably due to the greater mean age of patients in this group.

Discussion

A relationship between diabetic microangiopathy and an HLA antigen (or antigens) of the A, B, and/or C loci has been proposed by several authors,⁷⁻¹⁴ whose findings are, however, contradictory owing to the heterogeneity of populations under consideration, the small numbers of patients studied, together with the absence or lack of adequate control data. Barbosa

*et al.*¹⁴ reported a comparison of HLA antigen frequencies in large numbers of type 1 diabetics (aged less than 40 years at diagnosis of diabetes) with and without proliferative retinopathy. The frequency of B7 was significantly lower in proliferative retinopathies (7%) than in diabetics without proliferative retinopathy (20%), and our own findings are remarkably consistent (5% in proliferative retinopathies versus 17% in nonretinopathies.) None of our 27 patients with proliferative retinopathy and diabetic nephropathy (group D) were B7 positive. A similar underrepresentation of HLA B7 was noted in type 1 diabetic proliferative retinopathies by Standl *et al.*¹⁵ Nevertheless we must concede that the difference in frequency of B7 between proliferative retinopathies and nonretinopathies is modest, that it has not been noted in other studies, and that the aetiology of the putative protective influence of B7 remains obscure. We could not demonstrate a significant relationship between B7 and insulin antibody binding, although we have confirmed the association between high insulin antibody binding and the B15 positive, B8 negative genotype.¹⁶⁻¹⁸ Retinopathies and nonretinopathies had similar insulin antibody binding by serum in accordance with some¹² but not all¹⁹ previous studies. It remains to be seen whether HLA D/DR typing will clarify the issue concerning a possible genetic predisposition to diabetic microangiopathy. Preliminary data^{20,21} suggest there is an association between HLA DR4 and diabetic retinopathy. The weak association between retinopathy and absence of HLA B7 may be the consequence of a yet stronger relationship between retinopathy and DR4.

We have shown retinopathies to have significantly higher HbA_{1c} and plasma postprandial glucose concentrations than nonretinopathies. Our findings are therefore in accordance with those of Schanzlin *et al.*²² and West *et al.*²³ in type 1 and 2 diabetics respectively. These observations have little bearing on the controversial issue concerning the relationship between long-term diabetic control and the aetiology of diabetic microangiopathy. However, it is interesting to note that the glycaemic control of non-retinopathies is not particularly good, albeit better than that of the retinopathies.

Systolic hypertension has been shown²⁴ to precede the development of exudative retinopathy in middle-aged diabetic Pima Indians (type 2 diabetics). In the present study neither systolic nor diastolic hypertension was commonly found in type 1 diabetics, most of whom were aged less than 50 years, unless accompanied by diabetic nephropathy. In particular, the prevalence of hypertension was no higher in retinopathies without accompanying nephropathy than in nonretinopathies. It therefore seems likely that

hypertension is not a major aetiological factor in the development of severe diabetic retinopathy in type 1 diabetics. We have shown a strong relationship between hypertension and the association of proliferative retinopathy with diabetic nephropathy. While it seems probable that diabetic nephropathy was responsible for the development of hypertension in most cases, we cannot discount the possibility that hypertension was causally related to the development of nephropathy, or even retinopathy, in those patients with underlying diabetic nephropathy.

The present study confirms smoking to be more prevalent among retinopaths than nonretinopaths and is thus in accordance with the findings of Paetku *et al.*²⁵ However, this difference appears to be due to the remarkably high prevalence of smoking in proliferative retinopaths who have diabetic nephropathy. This finding is in agreement with the observations of Christiansen,²⁶ who reported heavy smoking to be a characteristic of diabetic nephropaths.

The present study suggests that the risk of development of diabetic microangiopathy is related not only to HLA associated genetic factors but also to cigarette smoking.

The authors gratefully acknowledge the skilled technical assistance provided by Judith Matthews, Margaret Sinclair, Dr A White, and C. Darg. We are also indebted to Miss L. Veitch for her invaluable secretarial assistance. The authors also gratefully acknowledge financial support provided by the British Diabetic Association. A.B.K. was further supported by a grant from Nordisk Insulin Laboratories.

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