Diabetologia (1999) 42: 1017–1020

Diabetologia

Short communication

Human leucocyte antigen and insulin gene regions and nephropathy in Type I diabetes

T.A. Chowdhury[1], **P.H. Dyer**[1], **C.H. Mijovic**[1], **D.B. Dunger**[2], **A.H. Barnett**[1], **S.C. Bain**[1]

[1] Department of Medicine, Birmingham Heartlands Hospital, Birmingham, UK
[2] Department of Paediatrics, John Radcliffe Hospital, Oxford, UK

Abstract

Aims/hypothesis. Diabetic nephropathy seems to have a strong genetic component. Genes involved in the genetic susceptibility to Type I (insulin-dependent) diabetes have been suggested to have a role in the development of diabetic nephropathy. This study aimed to examine the role of human leucocyte antigen and insulin genes in susceptibility to nephropathy in patients with Type I diabetes.
Methods. We carried out a genetic association study examining insulin gene polymorphisms using three large cohorts of patients with Type I diabetes: nephropathy (n = 258), long duration non-nephropathy (n = 153) and a recently diagnosed (sporadic) diabetic cohort (n = 264). Human leucocyte antigen typing results were obtained in a smaller number due to assay failures (n = 182, 126 and 200 respectively).
Results. No significant difference was seen in the distribution of human leucocyte antigen A, B, C, DR, *DQA1* and *DQB1* haplotypes and alleles between the three diabetic cohorts. No significant difference was seen in insulin ' + ' and '–' genotypes and alleles between the three diabetic cohorts.
Conclusion/interpretation. Human leucocyte antigen and insulin gene loci are unlikely to have a major role in the susceptibility to nephropathy in Caucasian patients with Type I diabetes in the United Kingdom.
[Diabetologia (1999) 42: 1017–1020]

Keywords Type I diabetes, diabetic nephropathy, human leucocyte antigen, insulin gene.

The human leucocyte antigen (*HLA*) region has a major influence on susceptibility to Type I diabetes [1] and is possibly implicated in susceptibility to diabetic complications. Human leucocyte antigen-identical, non-diabetic siblings of diabetic probands have glomerular basement membrane expansion, which is not observed in HLA-non-identical siblings [2]. This is also observed in *DR4* positive parents of diabetic patients, suggesting that even in the absence of diabetes, microangiopathy may occur due to HLA-related factors [2]. Previous examination of HLA in nephropathy has shown positive and negative associations with A2, B8, B15, DR4 and DR3/4 [3]. The insulin gene (*INS*) is also associated with Type I diabetes, and has been implicated in the onset of vascular disease [4]. As macrovascular disease is common in nephropathy, *INS* is a suitable candidate gene for susceptibility to nephropathy. Previous studies suggest an excess of class I allele homozygotes in patients with nephropathy [4].

Studies examining the role of HLA or *INS* have often been small and ascertainment of nephropathy status has been poor. The aim of our study was to clarify the role of these genes in susceptibility to nephropathy in three large, well characterised cohorts with Type I diabetes.

Received: 6 November 1998 and in final revised form: 31 March 1999

Corresponding author: Dr T. A. Chowdhury, Ward 24, Diabetes Centre, Birmingham Heartlands Hospital, Bordesley Green East, Birmingham, B9 5SS, UK
Abbreviations: HLA, human leucocyte antigen; *INS*, insulin gene; LDNN, long duration non-nephropathy.

Table 1. Characteristics of patients with Type I diabetes in the three diabetic cohorts

	Nephropathy (n = 258)	LDNN (n = 153)	Sporadic (n = 264)
Age at venesection (years)	39.2 [7.6]	37.8 [6.2]	18.01 [6.6]
Sex (M/F)	140/118	71/82	135/129
Age at diagnosis (years)	13.7 [6.2]	12.4 [8.4]	12.3 [5.9]
Duration of diabetes (years)	27.6 [8.0]	26.6 [8.7]	4.6 [4.3]
HbA_{1c} (%)	8.4 [2.1]	8.3 [1.9]	–
Systolic blood pressure (mm Hg)	160 [6]	132 [14]	–
Diastolic blood pressure (mm Hg)	95 [8]	72 [6]	–
On anti-hypertensive therapy (%)	89.5	12.3	–
Serum cholesterol (mmol/l)	5.8 [1.3]	5.6 [1.1]	–
Serum creatinine (μmol/l)	184 [36]	97 [22]	–

Data are means ± [SD]

Subjects and methods

Subjects. The patients in this study have been previously described [5]. Three white Caucasian Type I diabetic cohorts were typed. The nephropathy cohort *(n = 258)* had persistent "Albustix" positive proteinuria in the absence of other causes, retinopathy and hypertension. Recently diagnosed Type I diabetic patients were typed (sporadic diabetic group n = 264), as were patients with Type I diabetes longer than 20 years without proteinuria [long duration non-nephropathy (LDNN) group n = 153].

–23Hph1 INS typing. The *–23Hph1* polymorphism of *INS* has an A/T transversion at position 23, giving rise to a variable restriction site used to visualise class I and class III alleles. Genotyping was done using the Taqman LS-50B Detection System (PE, Applied Biosystems, Norwalk, Conn., USA), using fluorogenic probes *INS*-AT1 (5′-FAM-CCTGCCTGTCTCCCAGATCACT-TAMRA-3′) and *INS*-AT2 (5′-TET-CCTGCTG TCACCCAGATCACT-TAMRA-3′) and the primers *INS*-T1 (5′-CAAGCAG GTCTGTTCCAAGG-3′) and *INS*-T2 (5′-GGTCTTGGGTGTGTAGAA GAAGC-3′). This method obviates the need to do post PCR processing. 67% of *INS* types obtained in this way were confirmed using conventional restriction fragment length polymorphism (RFLP) analysis. The reaction volume of 50 μl contained 2 μl of each probe (2 $pmol.ml^{-1}$), 5 μl of 10 × buffer (Perkin Elmer, Beaconsfield, UK), 15 mmol/l magnesium chloride (Bioline, London, UK), 10 μl of 50% glycerol, 4 nmol/l of each dNTP (Pharmacia, Biotech, Uppsala, UK), 1 unit of Taq polymerase (Perkin Elmer), 2 pmoles of each primer and 100 ng of genomic DNA. Two stage PCR conditions were 94 °C for 1 min and 63 °C for 15 s for 40 cycles. Following PCR, the products were transferred to a viewing plate and fluorescence was read by the Taqman LS-50B luminescence spectrophotometer (PE Applied Biosystems). Genotypes were reported in comparison to samples with known genotypes homozygous for the + allele and – allele.

HLA typing. Not all samples could be HLA typed due to a large number of assay failures secondary to heparinisation, which can inhibit HLA typing [6]. We typed 200 sporadic, 126 LDNN and 182 nephropathy patients, using the phototyping method [7] which involves typing for *HLA-A*, *B*, *C*, *DR*, *DQA1* and *DQB1* using a mix of 144 sequence specific primers. The reaction volume of 13 μl contained 67 mmol/l Tris base pH 8.8, 16.6 mmol/l ammonium sulphate, 2 mmol/l magnesium chloride (Bioline), 0.01% (v/v) Tween 20, 100 ng of genomic DNA, 200 μmol/l of each dNTP (Pharmacia), 1–4 μmol/l of each allele specific primer, 0.1 μmol/l of control primer (primers 63 and 64), 1.3 μl of 10 × Buffer (Bioline) and 0.125 units of Taq polymerase (Bioline). We added 8 μl of buffer, DNA and enzyme mix to the 5 μl primer mix. PCR conditions were 1 min denaturation at 96 °C, 96 °C for 25 s, 70 °C for 45 s, 72 °C for 30 s (5 cycles), 96 °C for 25 s, 65 °C for 45 s, 72 °C for 30 s (21 cycles), and finally 96 °C for 25 s, 55 °C for 50 s, 72 °C for 90 s (4 cycles). Products were visualised on 0.8% agarose gels (FMC, Rockland, Main, USA).

Statistical analysis. Statistical analysis of *INS* and HLA genotypes was done using χ^2 analysis with Bonferoni correction for the number of tests done.

Results

No significant difference was seen between the LDNN and nephropathy groups in HbA_{1c}, age, duration of diabetes, serum cholesterol and age at diagnosis (Table 1). Significant higher serum creatinine, systolic blood pressure, diastolic blood pressure, and proportion of males were seen in the nephropathy group ($p < 0.01$).

For the sake of brevity, results of *HLA-A*, *B* and *C* typing in the three diabetic cohorts are not shown. No significant heterogeneity between the three cohorts in allele distribution was seen in any of the *HLA-A*, *B* and *C* alleles, including the alleles *A2*, *B8* and *B15* which had previously shown association.

No heterogeneity between the three cohorts in *DR* genotype was seen (Table 2). The *DR3/4* genotype was not over-represented in the nephropathy group compared with the sporadic ($\chi^2 = 0.549$, 1 *df*, $p = 0.459$), nor the LDNN ($\chi^2 = 1.160$, 1 *df*, $p = 0.282$) groups. The *DR4* allele was not over-represented in the nephropathy cohort compared with the sporadic ($\chi^2 = 0.003$, 1 *df*, $p = 0.954$) or LDNN ($\chi^2 = 0.103$, 1 *df*, $p = 0.749$) groups. No over-representation of *DQB1* or *DQA1* alleles in the nephropathy group was seen compared with the sporadic or LDNN groups (Table 2), although the *DQB1*0602* allele was slightly more prevalent in the nephropathy cohort, but the numbers were small and not statistically significant. The HLA haplotype distribution (*DR-DQA1-DQB1*) was compared between the

Table 2. HLA alleles, haplotypes and *INS* genotypes and alleles in the three diabetic cohorts

HLA region Allele		Sporadic (200)	LDNN (126)	Nephropathy (182)	*p* value (corrected)
DR	X/X	16 (8.0)	19 (15.1)	19 (10.4)	0.130
	3/X or 3/3	41 (20.5)	25 (19.8)	35 (19.2)	0.953
	4/X or 4/4	82 (41.0)	42 (33.3)	76 (41.8)	0.276
	3/4	61 (30.5)	40 (31.8)	52 (28.6)	0.827
DQB1	* 0201	130 (32.5)	96 (38.1)	126 (34.7)	0.344
	* 0301	46 (11.5)	22 (8.7)	36 (9.9)	0.506
	* 0302	115 (28.8)	81 (32.2)	116 (31.9)	0.550
	* 05	59 (14.8)	34 (13.5)	46 (12.7)	0.694
	* 0602	3 (0.8)	1 (0.4)	8 (2.1)	0.077
	* 0603	9 (2.2)	5 (1.9)	8 (2.0)	0.977
	Other	38 (9.5)	13 (5.2)	24 (6.6)	0.092
DQA1	* 0101	54 (13.5)	30 (11.9)	42 (11.6)	0.687
	* 0301	164 (41.0)	102 (40.5)	145 (39.8)	0.948
	* 05	118 (29.5)	73 (28.9)	108 (29.7)	0.982
	Other	64 (16.0)	47 (18.7)	69 (18.9)	0.522
HLA haplotype *DR-DQA1-DQB1*					
* 0101-0101-0201		13 (3.5)	10 (4)	16 (4)	0.946
* 0101-0101-04		2 (0.5)	2 (0.7)	2 (0.5)	0.885
* 0101-0101-05		35 (9.6)	24 (9.5)	41 (10.2)	0.939
* 0103-0101-05		3 (0.8)	0 (0)	4 (1.0)	0.301
* 0304-0201-0201		7 (1.9)	2 (0.7)	3 (0.7)	0.263
* 0304-03011-0201		28 (7.7)	22 (8.7)	36 (9.0)	0.798
* 0304-0502-0201		42 (11.5)	25 (10)	57 (14.2)	0.230
* 0401-0102-0604		54 (14.8)	33 (13.1)	42 (10.5)	0.195
* 0401-0201-0201		22 (6.0)	23 (9.1)	28 (7.0)	0.295
* 0401-03011-0201		67 (18.4)	51 (20.2)	84 (21.0)	0.660
* 0401-03011-0302		30 (11.9)	18 (7.1)	37 (9.25)	0.636
* 0401-03011-04		4 (1.1)	2 (0.8)	6 (1.5)	0.707
* 0401-03011-05		2 (0.5)	0 (0)	6 (1.5)	0.088
* 0701-0102-0201		4 (1.1)	2 (0.8)	0 (0)	0.126
* 0701-0201-0201		2 (0.5)	4 (1.6)	8 (2.0)	0.217
* 0302-0102-0201		4 (1.1)	0 (0)	6 (1.5)	0.162
		Sporadic (264)	LDNN (153)	Nephropathy (258)	*p* value (overall)
INS genotype	+/+	192 (72.7)	111 (72.8)	178 (69.0)	0.584
	+/–	61 (23.1)	38 (24.8)	65 (25.2)	
	–/–	11 (4.2)	4 (2.6)	15 (5.8)	
INS allele	+	0.84	0.85	0.82	0.357
	–	0.16	0.15	0.18	

Data are *n* (%) or proportion
X = non 3 or 4 allele
Only haplotypes with more than two subjects shown

three groups (Table 2). No significant heterogeneity between the three groups was seen. As our assay was unable to distinguish between *DRB1**0401–22 alleles, we were unable to analyse *DR4* subtypes according to *DQA1/DQB1* haplotypes.

The –23Hph ' + ' allele is equivalent to the class I allele and the '- ' allele is equivalent to the class III allele in Caucasian populations. There was no overall heterogeneity between the groups in *INS* genotypes ($\chi^2 = 2.847$, 4 *df*, $p = 0.584$). Genotype frequencies did neither differ between the sporadic diabetic group and the nephropathy group ($\chi^2 = 1.203$, 2 *df*, $p = 0.548$), nor the LDNN group ($\chi^2 = 2.3051$, 2 *df*, $p = 0.316$). No excess in either the ' + ' or '-' allele was seen in the nephropathy group compared with the sporadic group ($\chi^2 = 1.336$, 1 *df*, $p = 0.248$), nor the LDNN group ($\chi^2 = 1.543$, 1 *df*, $p = 0.215$). Consistent with this, no overall heterogeneity in allele frequency was seen between the three groups ($\chi^2 = 2.061$, 2 *df*, $p = 0.357$). The distribution of *INS* class I and II alleles according to HLA haplotype was analysed, and again no significant heterogeneity was noted (data not shown).

Discussion

This study is the largest examining *HLA* and *INS* genes in nephropathy in Type I diabetes. The results are consistent with previously observed data of the prevalence of diabetes susceptibility and protective alleles in Type I diabetic groups. The *DQB1**0302

and *0201 alleles were highly prevalent in all three diabetic groups, whereas the *DQB1**0602 and *0603 alleles were uncommon. Furthermore, over 95% of patients in the diabetic groups had high risk HLA-*DR3* or 4 alleles. Similarly, on examination of the *INS* region, overall 77.4% of the diabetic cohort had homozygosity for class I alleles which is similar to that seen in previously reported Caucasian populations with Type I diabetes.

Several small studies have examined HLA and *INS* in diabetic complications. These have been reviewed and have shortcomings of small numbers and poor patient characterisation [3]. Unsurprisingly, a number of contradictory results have been reported. Recently, higher prevalence of *HLA-DQB1**0201/0302 genotype has been suggested in patients with severe retinopathy compared with those free of retinopathy [8], and the presence of *HLA*-A24 has also been suggested to be a risk factor for early onset retinopathy, possibly due to more rapid loss of beta-cell function associated with this allele. Whilst hyperglycaemia is important in the pathogenesis of nephropathy, early beta-cell failure is probably not a major influence on the development of nephropathy as patients with nephropathy appear to be more insulin resistant than their non-nephropathic counterparts.

Larger studies of HLA in nephropathy have been reported recently. A study examined 24 patients with diabetic nephropathy and 51 patients with long duration of diabetes and no nephropathy and showed a significant protective effect of the *DR4* allele ($p = 0.02$) [9]. In a similar study, *INS* and HLA regions were examined in 26 patients with Type I diabetes and microalbuminuria and 30 subjects matched for age, sex and HbA_{1c} who were normoalbuminuric [10]. No significant difference in *INS* genotypes and alleles was noted, and there was no association with HLA. The cohort studied in the Wisconsin Epidemiologic Study of Diabetic Retinopathy has also been examined for genetic factors involved in diabetic nephropathy [4]. The study assessed 324 of the original cohort of 996 for nephropathy status. It was noted at initial examination that subjects with proteinuria were more likely to be class I homozygotes (I/I – 19% vs I/III or III/III – 10%, $p < 0.05$), although the nephropathy cohort was small ($n = 50$).

In this study no association between *INS*, *HLA-A*, *B*, *C*, *DR*, *DQA1* and *DQB1* alleles and nephropathy was seen on examination of large, well characterised cohorts of patients with Type I diabetes with and without nephropathy. In particular, the previously reported positive and negative associations with *HLA A2*, *B8*, *B15*, *DR3/4*, *DR4* and *INS* class I alleles could not be confirmed. No association with *HLA* haplotypes was noted. It is unlikely, therefore, that *HLA* and *INS* modulate the genetic influence in diabetic nephropathy in Caucasian subjects with Type I diabetes in the United Kingdom.

Acknowledgements. We are grateful to S. Marshall, S. Gibson, S. Davies, M. Nattrass, J. Dean, J. Adu, P. Mackin, P. Morris, J. Webber and S. Kumar who aided us with the collection of patients and to J. Heward who did the HLA typing. This study was supported by a grant from the Peel Medical Research Trust and the West Midlands NHS Executive Research and Development Fund.

References

1. She JX (1996) Susceptibility to Type I diabetes:HLA-DQ and DR revisited. Immunology Today 17: 323–329
2. Barbosa J, Chavers B, Steffes M et al. (1980) Muscle extracellular membrane immunofluorescence and HLA as possible markers of prediabetes. Lancet 1: 330–333
3. Barbosa J, Saner B (1984) Do genetics factors play a role in the pathogenesis of diabetic microangiopathy? Diabetologia 27: 487–492
4. Raffel LJ, Vadheim CM, Roth M-P, Klein R, Moss SE, Rotter JI (1991) The 5' insulin gene polymorphism and the genetics of vascular complications in Type I (insulin-dependent) diabetes mellitus. Diabetologia 34: 680–683
5. Chowdhury TA, Dronsfield MJ, Kumar S et al. (1996) Examination of two genetic polymorphisms within the renin-angiotensin system:no evidence for an association with nephropathy in IDDM. Diabetologia 39: 1108–1114
6. Satsangi J, Jewell DP, Welsh K, Bunce M, Bell JI (1994) Effect of heparin on polymerase chain reaction. Lancet 343: 1509–1510
7. Bunce M, O'Neill CM, Barnardo MCNM, Krausa P, Browning MJ, Morris PJ, Welsh KI (1995) Phototyping:comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilising sequence-specific primers (PCR-SSP). Tissue Antigens 46: 355–367
8. Agardh D, Gaur LK, Agardh E, Landin-Olsson M, Agardh CD, Lernmark A (1996) HLA-DQB1*0201/0302 is associated with severe retinopathy in patients with IDDM. Diabetologia 39: 1313–1317
9. Svejgaard A, Jakobsen BK, Platz P et al. (1986) HLA associations in insulin-dependent diabetes:search for heterogeneity in different groups of patients from a homogeneous population. Tissue Antigens 28: 237–44
10. Ronningen KS, Bangstad HJ, Undlien DE, Thorsby E (1993) Influence of genetic factors (HLA class II genes, insulin-gene region polymorphisms) and metabolic control on the development of diabetic nephropathy. Diabetes Research 23: 31–40