The HLA-D associations of Type 1 (insulin-dependent) diabetes in Punjabi Asians in the United Kingdom

O. Odugbesan, J. Fletcher, C. Mijovic, E. Mackay, A. R. Bradwell and A. H. Barnett

Departments of Medicine and Immunology, University of Birmingham and East Birmingham Hospital, Birmingham, UK

Summary. Type 1 (insulin-dependent) diabetes is less common in Asian Indians than in white Caucasoids. Forty-five Punjabi Asians with Type 1 diabetes and 96 racially matched control subjects were HLA-DR typed. DR3 was increased in diabetic patients vs control subjects (82% vs 38%, $p < 10^{-5}$) with relative risk 7.7 and etiological fraction 0.72. DR4 was increased in diabetic patients vs control subjects (31% vs 7%, $p < 0.003$) with relative risk 5.7 and etiological fraction 0.26. DR2 showed a negative association (relative risk 0.19, etiological fraction -0.28), as did DR7 (relative risk 0.21, etiological fraction -0.33). HLA-DQ β -chain gene probing using restriction enzyme BamHI in 43 diabetic patients and 90 control subjects showed that the DR1-associated 6.2 and 3.2kb fragments were less common in diabetic patients than in the control subjects (12% vs 36%, $p < 0.02$). A 12kb fragment was associated with DR4 in both diabetic patients and

control subjects. DR3 is the major susceptibility factor for Type 1 diabetes in Punjabi Asians and DR4 is a second marker. Gene probing indicates that the same DR4 subset is associated with the condition as in white Caucasoids. DR1 and its associated DQ β restriction fragments are reduced in Asian Type 1 diabetic patients making it unlikely that DR1 haplotypes carry a predisposing factor in this racial group. We conclude that the genetic component of Type 1 diabetes in Punjabis shows differences from that of the white Caucasoid population and that the lower frequency of DR4 in this population may contribute to the lower prevalence of Type 1 diabetes.

Key words. Type 1 (insulin-dependent) diabetes, Punjabi Asians, HLA-DR typing, DQ β -gene probing.

Studies of diabetes in India suggest that Type 1 (insulin-dependent) diabetes is considerably less common than in the United States or Europe [1–4]. In our clinic in Birmingham, United Kingdom only 4% of Asian diabetic patients have classical ketosis-prone, insulin-dependent diabetes compared with 18% of white Caucasoid diabetic patients [5]. On the other hand, the Southall UK survey [6] indicated that the prevalence of Type 2 (non-insulin-dependent) diabetes in the Asian population in the UK is substantially higher than in white Caucasoid subjects. The lower prevalence of Type 1 diabetes in Asian Indians may be due either to different genetic or environmental factors or both. We have studied the HLA-D region by both serological and gene probing techniques in Punjabi Asians living in the UK to determine whether the genetic factors responsible for the predisposition to Type 1 diabetes differ from those in white Caucasoid subjects.

Subjects and methods

Unrelated Asian Indian subjects with Type 1 diabetes attending 7 diabetic clinics in the West Midlands (Birmingham area of the UK)

were studied. Type 1 diabetes was defined as ketosis-prone diabetes with acute onset of symptoms, absolute requirement for insulin from diagnosis and age at diagnosis less than thirty years. Healthy racially matched unrelated control subjects with no family history of diabetes were recruited from antenatal clinics and community centres. In all cases the subjects or both their parents were born in the Punjab region of the Indian subcontinent. Seventeen of the 45 diabetic patients were born in the UK and the remainder in the Punjab. In all diabetic patients, the initial diagnosis of Type 1 diabetes was made in the UK. One of the diabetic patients had autoimmune hypothyroidism, but there were no other patients with autoimmune polyendocrinopathy.

HLA-DR typing was performed in 45 Type 1 diabetic patients and in 96 control subjects. Southern blot hybridization analysis was performed in 43 diabetic patients and in 90 control subjects. Both analyses were performed in 43 diabetic patients and in 79 control subjects. For technical reasons the remaining subjects had only one of these investigations performed. Peripheral blood lymphocytes were enriched for B cells by depletion of T cells using sheep erythrocyte rosetting. HLA-DR antigens 1–5 and 7 were identified by a standard microlymphocytotoxicity method [7] using commercially prepared typing trays (Biotest (UK), Birmingham, UK). Seven antisera were available for each of the specificities DR1–5 and five antisera for DR7.

DNA was isolated from peripheral blood of Type 1 diabetic patients and control subjects. 10 μ g DNA was digested with restriction

enzyme BamHI under conditions recommended by the manufacturer (BRL, Glasgow, UK). Digested DNA was separated by electrophoresis in 0.7% agarose at 50 V, 25 mA for 18–22 h and blotted by the method of Southern onto nitrocellulose filters [8]. The HindIII/PstI insert of pII- β -1 (DQ β chain) (kindly provided by P.A. Peterson, Uppsala, Sweden), was labelled to a specific activity of 10^9 cpm/ μ g DNA by the oligonucleotide primer method [9]. Prehybridization and hybridization were performed at 65 °C in $6 \times$ standard sodium citrate solution (SSC; $1 \times$ SSC = 0.15 mol/l NaCl, 0.015 mol/l Na citrate), $5 \times$ Denhardt's solution, 0.5% sodium dodecyl sulphate (SDS) with 250 μ g/ml denatured salmon sperm DNA. Ten percent dextran sulphate was used in the hybridization solution. After hybridization, filters were first rinsed in $2 \times$ SSC, 0.1% SDS, twice, at room temperature, then washed in the same solution at 65 °C for 30 min. Washing was then performed in $0.5 \times$ SSC, 0.1% SDS at 65 °C for 60 min, followed by four 15-min washes in $0.1 \times$ SSC, 0.1% SDS at 65 °C. After air drying, autoradiography was performed for 3 days at –70 °C.

Statistical analysis

The chi-squared test was used where all cells in a 2×2 contingency table had expected values greater than five. Otherwise, Fisher's exact test was employed. The p values were corrected (p_c) by multiplying by the number of DR antigens tested. Relative risk (RR, a measure of the increased risk of developing the disease if exposed to a particular risk factor) and etiological fraction (EF, the proportion of the disease in the population that is associated with the risk factor) were calculated by standard methods [10].

Results

Results of HLA-DR typing are shown in Table 1. There was a strong positive association between DR3 and Type 1 diabetes with 82% of diabetic patients possessing DR3 in comparison with 38% of control subjects ($p_c < 10^{-5}$, RR 7.7, EF 0.72). DR4 was also significantly increased in diabetic patients compared with control subjects (31% vs 7%, $p_c < 0.003$), with RR 5.7 and EF 0.26. Ninety-one per cent of diabetic patients possessed DR3 and/or DR4 compared with 42% of control subjects ($p_c < 10^{-7}$, RR 14.4, EF 0.85). DR 3, 4 heterozygosity was increased in diabetic patients (22%) compared with control subjects (3%) ($p_c < 0.007$, RR 8.9, EF 0.20). DR2 and DR7 showed negative associations with Type 1 diabetes, being more frequent in control subjects than diabetic patients – DR2: 27% vs 7%, $p_c < 0.04$, RR 0.19, EF –0.28; DR7: 31% vs 9%, $p_c < 0.03$, RR 0.21, EF –0.33. DR1 was less common in diabetic patients than in control subjects but this difference was not significant. None of the diabetic patients was a DR1, 4 heterozygote.

HLA-DQ β chain gene probing using the enzyme BamHI produced a pattern of approximately 10 distinct fragments. The patterns obtained in the Asians were similar to those obtained in white Caucasoid subjects, with no fragment unique to Asians or to white Caucasoid subjects being identified. The frequencies of 12, 3.7 and 3.0 kb fragments and of a 6.2 kb fragment (which is always associated with a 3.2 kb fragment) in the patient and control groups are given in Table 2.

Table 1. HLA-DR antigen frequencies in Punjabi Asians in the UK

HLA antigen	Type 1 diabetic patients ($n=45$)	Control subjects ($n=96$)	RR	EF	Pc
	No. (%)	No. (%)			
DR1	2 (4)	10 (10)	0.4	–0.07	NS
DR2	3 (7)	26 (27)	0.19	–0.28	<0.04
DR3	37 (82)	36 (38)	7.7	0.72	< 10^{-5}
DR4	14 (31)	7 (7)	5.7	0.26	<0.003
DR5	4 (9)	17 (18)	0.45	–0.11	NS
DR7	4 (9)	30 (31)	0.21	–0.33	0.03
DR3, 4	10 (22)	3 (3)	8.9	0.20	0.007
DR3 and/or 4	41 (91)	40 (42)	14.4	0.85	< 10^{-7}

No. (%) = number (percentage) of individuals with the corresponding DR phenotype; RR = relative risk; EF = etiological fraction; Pc = significance level corrected for number of markers tested

Table 2. HLA-DQ beta chain RFLPs in Punjabi Asians in the UK

Fragment (kb)	Type 1 diabetic patients ($n=43$)	Control subjects ($n=90$)	RR	Pc
	No. (%)	No. (%)		
12	16 (37)	23 (26)	1.73	NS
6.2 + 3.2	5 (12)	32 (36)	0.24	<0.02
3.7	5 (12)	20 (22)	0.46	NS
3.0	1 (2)	18 (20)	0.10	0.03

No. (%) = number (percentage) of individuals with the fragment; RR = relative risk; Pc = significance level corrected for number of fragments tested

Associations were noted between restriction fragments and certain DR specificities in the control subjects: 12 kb fragment and DR4 ($p < 10^{-3}$) (all 7 DR4 positive controls were 12 kb positive), 12 kb fragment and DR7 ($p < 5 \times 10^{-4}$); 6.2 + 3.2 fragments and DR1 ($p < 0.005$); 3.7 kb fragment and DR5 (w11) ($p = 10^{-6}$); 7.0 + 4.0 kb fragments (not shown in Table 2) and DR3 ($p < 0.0005$). No association of the 3.0 kb fragment was apparent.

The 12 kb fragment was strongly associated with DR4 in patients – all 14 DR4 positive patients studied were 12 kb positive, but this was the case in only three out of 29 DR4 negative patients ($p < 10^{-7}$). None of the DR4 positive patients possessed the 3.7 kb fragment. All 35 DR3 positive patients studied possessed the 7.0 + 4.0 kb fragments.

The combination of 6.2 and 3.2 kb fragments was less common in diabetic patients than in control subjects (12% vs 36%, $p_c < 0.02$). The RR for the 6.2 + 3.2 kb fragment combination was 0.24. A 3.0 kb fragment was found in 20% of control subjects but in only 2% (one out of 43) of diabetic patients ($p_c < 0.03$).

Discussion

Asian Indians can be divided into the Dravidians, mainly living in the south, and those of Indo-Aryan stock who live in the north. The UK Asian community mainly originates from India, Pakistan, Bangladesh and Sri Lanka. Asians living in Birmingham, UK are mainly from Gujarat or the Punjab. We have restricted our study to individuals from the Punjab (an area spanning northeast Pakistan to northwest India) to minimise the influence of genetic heterogeneity.

The major component of genetic susceptibility to Type 1 diabetes is HLA-linked. In most white Caucasoid populations both HLA-DR3 and DR4 are strongly associated with Type 1 diabetes so that over 95% of diabetic patients possess either or both of these markers [11, 12]. At least 50% of healthy white Caucasoid subjects possess either or both of these markers, however, and this has led to a search for more specific markers for susceptibility to Type 1 diabetes.

Our results indicate that DR3 has a strong positive association with Type 1 diabetes in Punjabi Asians with RR 7.7 and EF 0.72. DR4 is also significantly associated, but with a lower RR and EF. There is a very low frequency of DR4 in the control population. DR2 and DR7 are markedly reduced in diabetic patients, suggesting that these markers are associated with a protective effect.

There are few published studies on DR antigens in Asian Indians with Type 1 diabetes. A study of Indians with Type 1 diabetes in South Africa, however, showed an association with DR3 and DR4 in Aryans but, presumably because of the small number of patients studied, no DR association in Dravidians [13]. Bhatia et al. found a strong association with DR3 but not with DR4 in north Indian Type 1 diabetic patients [14]. The frequency of DR4 in their control population, however, was higher than that found in other studies [15, 16]. The stronger association of DR3 than of DR4 with Type 1 diabetes in Punjabi Asians contrasts with findings in the UK white Caucasoid population where DR4 has a higher RR and EF than DR3 [12]. In both groups, however, DR2 and DR7 show a negative association. The finding of a high frequency of DR1/4 heterozygotes in white Caucasoid diabetic patients [17] suggests that DR1 haplotypes may predispose to Type 1 diabetes. DR1 was, however, reduced in frequency in our diabetic patients, but this difference was not significant.

The HLA-D region comprises three sub-loci – DP, DQ and DR. The molecules encoded in this region are thought to be important in interactions between cells of the immune system [18]. The DP and DQ subregions each contain two α and two β chain genes, while the DR region has one α chain and, usually, three β chain genes. Until recently cellular and serological typing were the only methods for studying HLA-D region polymorphism. The development of the technique of

Southern blot hybridization analysis and the isolation of cDNA clones for HLA-D α and β chains has made it possible to study the variability of this region by restriction fragment length polymorphism (RFLP) analysis. This method has been employed in the study of Type 1 diabetes in white Caucasoid subjects with a variety of HLA-D region gene probes and restriction enzymes [19–23]. Owerbach et al. showed that a probe detecting DQ β DNA sequences could differentiate DR-matched patients and control subjects [20]. A BamHI 3.7kb fragment, found in 30–40% of control subjects, was rare (0–2%) in Type 1 diabetic patients. Subsequent family studies indicated that the 3.7kb fragment is a marker for a subset of DR4 chromosomes which is reduced in diabetic patients, and that the Type 1 diabetes-predisposing subset is associated with a 12kb BamHI fragment [19]. DR4 is in strong linkage disequilibrium with DQw3 in white Caucasoids, but DQw3 is also found in association with DR5 and on some haplotypes expressing DR7. DQw3 can be split serologically by reagents which define a specificity designated TA10 [24]. DR5–DQw3 haplotypes are TA10-positive, while the majority of DR4–DQw3 haplotypes are TA10-negative. Kim et al. have reported, in studies of RFLPs in homozygous typing lines, that the BamHI 12kb fragment is found in DQw3, TA10-negative (DQw3.2) cells, and the 3.7kb fragment in DQw3, TA10-positive (DQw3.1) cells [25]. DR4 haplotypes can thus be divided into DQw3.1 and DQw3.2 subsets. These workers have reported that it is the DQw3.2 allele which is associated with Type 1 diabetes and similar results have been obtained by others [21–23].

The DR4 haplotypes in our diabetic patients are of the DQw3.2 subset which predisposes to Type 1 diabetes in white Caucasoid subjects. The number of DR4-positive control subjects in our study is too small to determine accurately what proportion of DR4 haplotypes in the general population is of this type. The BamHI 12kb fragment was also associated with DR7 in the control subjects, but there was only one DR7/12kb-positive individual amongst the Type 1 diabetic patients. This suggests that some component other than the DQ β chain, present on DR4 haplotypes, but not on DR7 haplotypes, is influencing susceptibility. In this study, DR3, both in diabetic patients and control subjects, was associated with one fragment pattern (7.0 + 4.0kb fragments) and, therefore, DQ β chain gene probing could not specify any DR3 subset predisposing to Type 1 diabetes. A 6.2 + 3.2kb fragment combination was significantly less common in diabetic patients than in control subjects. This fragment pair is associated with DR1 both in our study and in white Caucasoid subjects [23]. Although DR1 haplotypes may predispose to Type 1 diabetes in white Caucasoid subjects [17], in our study this pattern is associated with a protective effect. The 3.0kb fragment, which was not found in any of the patients, did not show any DR as-

sociations in our study, but it may be associated with DRw6 [23] which was not tested for in this study.

We conclude that the major susceptibility factor so far defined for Type 1 diabetes in Punjabi Asians is HLA-DR3 with DR4 being a second predisposing factor and DR2 and DR7 protective. Gene probing indicates that the same subset of DR4 haplotypes, defined by DQ β restriction fragments, is associated with Type 1 diabetes in this racial group as in white Caucasoid subjects. DR1 and its associated DQ β restriction fragments were reduced in frequency in this group in contrast with the findings in white Caucasoid subjects. The low prevalence of Type 1 diabetes in Asian Indians remains unexplained, although the low frequency of DR4 may be partially responsible. It is likely, however, that environmental factors are also involved.

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Dr. A. Barnett
Department of Medicine
East Birmingham Hospital
Birmingham B9 5ST
UK