Factors governing the human immune response to injected insulin

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Summary. Seventy-nine patients were observed prospectively during their initial period of treatment with conventional bovine insulins. Insulin antibody levels 6 months after starting insulin therapy did not correlate with age, gender or β cell function at onset of treatment. Patients who required soluble insulin in addition to isophane insulin developed higher levels of insulin antibody. Patients bearing the HLA-B8, DR3 and C4AQO alleles had lower levels of insulin antibody, whereas those bearing DR7 produced significantly higher levels. Other alleles at the C4A, C4B, C2, factor B or Gm loci did not ap-

pear to have a significant effect on insulin antibody production. The hyporesponsiveness of B8/DR3/C4AQO-positive individuals probably reflects a non-specific abnormality of immunity whereas the enhanced responsiveness of those positive for DR7 suggests the presence of a specific immune response gene for insulin

Key words: Insulin, insulin antibody, immunogenicity, immune response genes, haemocyanin, HLA, DR7, C2, C4, factor B, Gm, C-peptide.

Diabetic individuals differ widely in their antibody response to injected insulin and there are likely to be several reasons for these variations in immunogenicity [1, 2]. Constitutional factors are clearly important as some patients fail to respond to the more immunogenic bovine preparations [3]. There have been few attempts to examine the relative contribution of these different variables but several retrospective studies of clinic populations of insulin-dependent diabetic patients suggest a link between low antibody response to insulin and possession of HLA-B8 and DR3 [4–6]. We decided, therefore, to mount a prospective study of new insulin-requiring diabetic patients treated with conventional bovine insulin preparations, to examine the importance of genetic and other factors in determining the magnitude of the immune response to insulin.

Subjects and methods

Subjects

Seventy-nine patients of Caucasoid origin, in whom a clinical decision had been made to start insulin treatment, were recruited to the Nottingham prospective study. There were 45 men and 34 women whose ages ranged from 14 to 82 years (mean 40 years). Classification of diabetes into Types 1 and 2 remains to matter of considerable dispute and we had difficulty in assigning some of the patients in this

study. It is easy to say that someone has Type 1 (insulin-dependent) diabetes if he or she presents in ketoacidosis but much more difficult to classify a young thin patient in whom diagnosis is made and treatment started before ketonuria has developed. For the purposes of the present study, we have categorised as Type 1 those patients who had had symptoms for less than 6 weeks and/or had ketonuria (more than ++ on Ketosticks: Ames, Indianapolis Indiana, USA) or had positive islet cell antibody. Patients with none of these features were designated Type 2 (non-insulin-dependent) unless they had some other cause of diabetes, e.g.: chronic pancreatitis or maturity-onset type diabetes of the young (MODY). On this basis, 54 patients had Type 1, 20 Type 2 and five had diabetes due to other causes. Of the 20 patients classified as Type 2, eleven were less than 60 years of age at diagnosis. Blood samples were taken before starting insulin (and in the presence of considerable hyperglycaemia) for determination of serum C-peptide concentrations and to confirm the absence of insulin antibody. All patients were treated with bovine insulin preparations purified by sequential recrystallisation (i.e. contaminated with ~1000 ppm proinsulin): 58 received isophane alone, 17 isophane with an acid soluble preparation and four lente insulin. Most patients were started on insulin as outpatients under the supervision of diabetes nurse practitioners. Soluble insulin was added if post-prandial hyperglycaemia was not controlled. Blood samples were taken for insulin antibody determination at 1, 3 and 6 months after commencing insulin therapy. Patients attended for a special visit between 3 and 6 months after entry when full HLA, C2, C4, factor B and Gm typing was performed.

Methods

IgG insulin antibody reactive with ¹²⁵I-labelled bovine insulin was measured as described previously [3]. Insulin was removed from sera prior to assay and results expressed in $\mu\text{g/l}$ without subtraction for

Fig. 1 A and B. Insulin antibody levels 6 months after starting insulin treatment: **A** arithmetical distribution, **B** log_e distribution

Fig. 2. Log-log plot of C-peptide levels before starting insulin treatment against insulin antibody levels occurring 6 months later

normal serum binding [7]. Circulating C-peptide levels were determined by radioimmunoassay and expressed in nmol/l [8]. Islet cell antibodies were sought by indirect immunofluorescence using cryostat sections of unfixed human group 0 pancreas [9]. HLA typing at A, B and C loci was performed using a standard microlymphocytotoxicity assay with reagents specific for the following alleles: A1, 2, 3, 9, 10 (25+26), 11, 28, 29, w30, w31, w32, w33; B5, 7, 8, 12 (w44+w45), 13, 14, 15, w16 (w38+w39), 17, 18, w21 (w49+w50), w22, 27, w35, 37, 40, w41, w47; Cw1, w2, w3, w4, w5, w6 and w7. DR typing was performed on B lymphocytes purified by E-rosette depletion [10] with reagents specific for DR1, 2, 3, 4, 5, w6, 7 and w9. C2 allotypes B, C and null were detected using isoelectric focussing with a haemolytic overlay [11]. The following C4 and factor B (Bf) allotypes were determined using high voltage electrophoresis in agarose with immunofixation: C4A – 1, 2, 3, 4, 5, 6 and QO, C4B – 1, 2, 3 and QO, Bf – F, F1, S and S1 [12, 13]. The nomenclature for C4 is as agreed at the Fourth International Complement Workshop [14]. Gm typing was performed by haemagglutination inhibition [15] using reagents specific for the following allotypic markers: IgG₁ (a, x, z and f), IgG₂ (n) and IgG₃ (b and g) obtained from the Central Laboratory, Dutch Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Statistical analysis

The statistical analysis used standard Student's t-test and analysis of variance techniques to identify subgroups with different mean anti-

Fig. 3. **A** Insulin antibody levels in patients who had acute symptoms for > 5 weeks versus < 5 weeks before starting insulin treatment. **B** Insulin antibody levels in patients treated with isophane insulin alone versus those requiring soluble insulin in addition

Table 1. Insulin antibody levels for patients possessing or lacking alleles detected at HLA-B and HLA-DR loci

Allele	Antibody levels (μg/l)						<i>p</i>
	Allele present			Allele absent			
	No. of patients	Log _e mean	Geometric mean	No. of patients	Log _e mean	Geometric mean	
HLA –B7	13	3.17 ± 0.26	23.8	66	2.90 ± 0.11	18.2	NS
B8	32	2.70 ± 0.15	14.9	47	3.11 ± 0.14	22.4	0.05
B12	25	3.16 ± 0.18	23.6	54	2.84 ± 0.13	17.1	NS
B15	23	2.95 ± 0.19	19.1	56	2.94 ± 0.13	18.9	NS
B18	5	2.41 ± 0.72	11.1	74	2.98 ± 0.10	19.7	NS
B40	9	3.25 ± 0.22	25.8	70	2.90 ± 0.12	18.2	NS
HLA –DR1	10	2.66 ± 0.27	14.3	69	2.99 ± 0.11	19.9	NS
DR3	36	2.72 ± 0.17	15.2	43	3.13 ± 0.13	22.9	< 0.05
DR4	50	2.97 ± 0.13	19.5	29	2.91 ± 0.17	18.4	NS
DR6	8	2.95 ± 0.30	19.1	71	2.94 ± 0.11	18.9	NS
DR7	14	3.60 ± 0.17	36.6	65	2.80 ± 0.12	16.4	0.002

Log_e results expressed as mean ± SEM. NS = not significant

body levels. Initial log_e transformation of the data was necessary to ensure that the assumptions implicit in these procedures were satisfied. Results are thus expressed in terms of log_e mean ± SEM, as well as geometric means on the original measurement scale.

Results

The distribution of insulin antibody levels 6 months after starting insulin therapy is strongly positively skewed (Fig. 1A), whereas log_e-transformation gives a fairly symmetrical distribution (Fig. 1B). Figure 2 is a log-log plot of C-peptide levels before starting insulin treatment against insulin antibody levels 6 months later and it shows the lack of relationship between these two parameters. There was also no relationship between age or

gender and the level of insulin antibody achieved. Accurate knowledge of duration of symptoms prior to starting insulin was available on 56 patients, of whom 28 had had symptoms for less than 5 weeks and 28 for longer than this (Fig. 3A). The difference between these two groups is not significant at the 5% level. Patients who required soluble insulin in addition to isophane generally showed a higher insulin antibody level ($p < 0.05$, Fig. 3B). The group numbers and insulin antibody levels for patients possessing each of the more common HLA-B and HLA-DR alleles detected in this population are set out in Table 1 and compared in each case with the remaining patients who lack each allele. Individuals bearing HLA-B8 and DR3 have lower insulin antibody levels ($p = 0.05$ and < 0.05 , respectively),

Table 2. Comparison of insulin antibody levels for DR7 positive versus negative individuals belonging to different groups of patients

Groups	Antibody levels (μg/l)						<i>p</i>
	DR7-Positive			DR7-Negative			
	No. of patients	Log _e mean	Geometric mean	No. of patients	Log _e mean	Geometric mean	
Patients with diabetes Type 1 (<i>n</i> = 54)	7	3.30 ± 0.22	27.1	47	2.77 ± 0.13	16.0	0.15
Patients with diabetes other than Type 1 (<i>n</i> = 25)	7	3.89 ± 0.21	48.9	18	2.89 ± 0.24	18.0	0.025
Whole group (<i>n</i> = 79)	14	3.60 ± 0.17	36.6	65	2.80 ± 0.12	16.4	0.002

Log_e results expressed as mean ± SEM**Table 3.** Insulin antibody levels for patients possessing certain phenotypes at the C2, factor B, C4A, C4B and Gm loci

		Antibody levels (μg/l)						<i>p</i>
		Phenotype present		Comparison group				
		No. of patients	Log _e mean	Geometric mean	No. of patients	Log _e mean	Geometric mean	
C2C	68	2.92 ± 0.11	18.5	C2B/C	10	3.15 ± 0.29	23.3	NS
Bf S	55	2.86 ± 0.12	17.5	Bf S/other ^b	24	3.13 ± 0.19	22.9	NS
C4AQO	5	1.91 ± 0.31	6.8	Others ^c	74	3.01 ± 0.11	20.3	<0.01
C4B1	43	3.06 ± 0.13	21.3	Others ^d	36	2.80 ± 0.16	16.4	NS
Gm fb	36	2.77 ± 0.14	16.0	} ^e				
fb/ag	32	3.07 ± 0.17	21.5					NS
ag	11	3.17 ± 0.31	23.8					
Gm n +	48	2.88 ± 0.13	17.8	n-	31	3.04 ± 0.18	20.9	NS
x +	18	3.12 ± 0.21	22.7	x-	61	2.89 ± 0.12	18.0	NS

Log_e results expressed as mean ± SEM. NS = not significant

^a The one remaining individual was C20; ^b these patients consisted of 20 S/F, 3 S/F1 and one S/S1; ^c these were: 51 A3, 11 A3/other, nine A4 and three miscellaneous; ^d these were: 13 B1/B3, 13 B1/B2 and 10 miscellaneous. ^e one-way analysis of variance failed to detect any difference between phenotype means

whereas those possessing DR7 show significantly higher levels of insulin antibody ($p = 0.002$). The presence or absence of DR4 did not correlate with insulin antibody levels. p values are for comparisons between groups possessing or lacking each allele. If the p value for the DR association is corrected for the number of alleles examined according to the method of Edwards [16] (i.e. $p_c = 1 - (1 - p)^n$ where $n = 5$) then p_c values for the DR3 and DR7 associations become, respectively, 0.23 and 0.01. The DR7 association is thus unambiguous but would not have been detected using the present data if patients with diabetes of other than Type 1 had been excluded (Table 2). Although the geometric mean for patients with Type 1 diabetes bearing DR7 is higher than for those who lack this allele, this difference is not statistically significant and it is not until all data are analysed that an association, significant after correction for the number of alleles examined, is established.

Table 3 lists antibody levels for patients bearing different phenotypes at the C2, C4, factor B and Gm loci. The only association observed is a link between possession of the C4AQO allele and a reduced antibody re-

sponse to insulin and this allele was present in association with HLA-B8 and DR3 in each case. Twenty-three patients possessed the DR3/DR4 phenotype and thus had a consistent genotype at the DR locus. These patients were examined separately for any effect of polymorphism at the complement or immunoglobulin allotype loci. No further associations were detected.

Discussion

The insulin antibody data obtained 6 months after starting insulin therapy (Fig. 1) confirm the wide heterogeneity of response and positively skewed distribution described previously [3, 17]. However, log_e transformation gives an acceptably symmetrical distribution and therefore we analysed the insulin antibody response as a continuous variable rather than compartmenting it into low and high categories with an arbitrary threshold in between. We have shown previously that most insulin antibodies induced by bovine insulin therapy are specific for determinants shared by the endogenous human

molecule [3]. There is evidence to suggest that the ease with which the natural state of tolerance to self proteins is broken is determined by the level of such proteins present in the circulation [18]. It therefore seemed important to investigate a possible relationship between the level of insulin antibody production and the level of β cell function at the time insulin therapy was instituted. However, we find no relationship between β cell function – as evidenced by serum C-peptide levels – and insulin antibody production 6 months thereafter (Fig. 2). Our patients covered a wide age range (although children were excluded) but there was no evidence of any association between insulin antibody production and age or gender. Patients varied considerably in their duration of symptoms of diabetes before starting insulin treatment but this did not have a significant effect on insulin antibody production. Those patients who required soluble insulin in addition to isophane did, however, show a significant enhancement of antibody production (Fig. 3). This is in keeping with the fact that soluble preparations of conventional bovine insulin also induce greater levels of antibody reactive with C-peptide and pancreatic polypeptide [19, 20].

The association of HLA-B8 and DR3 with low levels of insulin antibody production has been recorded previously [4–6], but these studies were retrospective and patients either had received a mixture of insulin species or the species used was not identified. Our finding of an association between HLA-DR7 and an increased level of insulin antibody production is new and rather more striking. Our policy was to include all patients started on insulin without restricting recruitment to those with Type 1 diabetes. Although HLA-DR3 and DR4 are much more common in Type 1 diabetes, HLA-DR2 and DR7 are much less common than in the general population [21] and the association with DR7 would not have been observed if we had restricted our study to patients with Type 1 diabetes (Table 2).

The C2, C4 and factor B loci lie between HLA-DR and HLA-B on chromosome 6. Possession of the allele C4AQO associated with low responses to insulin and all five patients with this allele possessed HLA-B8 and DR3 in addition. These alleles are positively associated and frequently co-exist as part of a common haplotype [22]. No other associations were observed between the magnitude of insulin antibody responses and individual alleles at the C2, C4 and factor B loci. The 23 DR3/DR4 heterozygotes in whom a consistent genotype had been established at the DR locus seemed an ideal subgroup in which to examine further the possible influence of variation at the C2, C4 and factor B loci. However, this too did not reveal any other significant associations.

Nakao et al. [23] reported an association between variation at the IgG Gm allotype locus on chromosome 14 and insulin antibody responsiveness. Gm typing in our study did not reveal a significant association for the group as a whole or the sub-population of 23

DR3/DR4 heterozygotes. However, the Gm data presented in Table 3 do show a consistent trend with *ag* homozygotes giving the highest mean value, *fb* homozygotes the lowest and *ag/fb* heterozygotes an intermediate value. The x positive group also gives a higher mean value than the group of x negative individuals. This result is in keeping with the overall finding of Nakao et al. [23] that the Gm 1, 2, 21 (i.e. *xag*) haplotype is associated with the *presence* of insulin antibody, although the cut off point which they used to distinguish between 'presence' and 'absence' of insulin antibody was not defined. Further studies are required to substantiate this association.

The DR7 association with elevated antibody responses to insulin appears to be independent of genes at the other HLA loci. However, a previous study on patients with insulin allergy [24] did describe an association with DR7, Bw44 and A2 but antibody levels were not reported and their patients were of mixed ethnic origin and diabetes type.

Immune response genes for insulin have been documented in mice and guinea pigs [25–27] although there is debate concerning their site of action [28]. It seems likely that DR7 identifies an immune response gene governing the human antibody response to injected insulin and the fact that the antibody response to an immunising injection of *Helix pomatia* haemocyanin (given to 47 patients from this same population) does not show a similar correlation is in support of this (unpublished observations). The immune response gene may be DR7 itself or a gene in linkage disequilibrium with it – possibly at the adjoining SB or DC loci [29]. DR3 may identify an immune suppression gene [30] although the association with a non-functional complement allotype (C4AQO) plus evidence that individuals bearing the B8/DR3 haplotype are less able to clear immune complexes, may indicate a non-specific abnormality of immunity [31].

HLA-DR7 associates with coeliac disease, dermatitis herpetiformis and persistent lymphocytosis [32–34] and further study of specific immune responses that associate with DR7 should tell us more about the pathogenesis of these and other DR-related diseases.

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