HLA-DR3 and DR7 in coeliac disease: immunogenetic and clinical aspects

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SUMMARY The association of HLA-A,B,C, DR polymorphisms and of Bf and GLO with coeliac disease was analysed in 100 Italian children. Primary involvement of HLA-DR3 and DR7 is apparent, while specificities of nearby loci are probably associated secondarily, because of linkage disequilibrium. Direct assessment of D/DR genotype through family studies and mixed lymphocyte cultures led to the recognition of two high risk genotypes DR3/3 and DR3/7, and of two lower risk genotypes DR3/X and DR7/X. The different weight of the HLA-dependent genetic factors is to some extent correlated with the clinical and immunological parameters, suggesting that the low-risk genotypes induce a milder expression of coeliac disease. Furthermore, other genetic factors, such as sex, appear to contribute to the penetrance of the disease, especially in the case of DR3/X and DR7/X.

Both genetic and environmental factors contribute to the onset and to clinical progression of coeliac disease. The role of genetic factors has long been substantiated by a high concordance rate in monozygotic twins, and an increased incidence of overt malabsorption or of asymptomatic histological lesions in first-degree relatives.^{1,2} More recently, the association between markers of the HLA system and coeliac disease has provided new evidence of the importance of genetic factors in this disease.³⁻⁸ Different genetic models have been put forward, which propose dominant or recessive expression of HLA-linked gene(s), or in addition assume the involvement of non-HLA gene(s).^{9,10}

In susceptible individuals, exposure to dietary gluten, or to purified gliadin fractions under experimental conditions, is the leading known environmental factor. Clinical and/or histological improvement on a gluten-free diet and relapse after gluten challenge have thus been established as fixed diagnostic criteria.¹¹ Nevertheless, the protean nature of the clinical picture is adequately shown by the significant proportion of atypical cases.¹²

As part of an ongoing study,⁶ the present report (1) provides further evidence of the association of

coeliac disease with HLA specificities; (2) investigates the heterogeneity of coeliac disease by comparing the available genetic markers with some clinical and immunological parameters.

Methods

PATIENTS

Thirty nine boys and 61 girls, unrelated and aged two to 18 years (including the 45 patients previously reported⁸), were studied. In all cases, diagnosis was based on the criteria established by the European Society for Pediatric Gastroenterology and Nutrition (ESPGAN):¹¹ (i) subtotal villous atrophy of the jejunal mucosa on gluten diet (first biopsy); (ii) clinical and histological improvement on gluten-free diet (second biopsy); (iii) histological relapse after gluten challenge (third biopsy). The personal and family history of each patient was recorded, with particular attention to breast feeding and its duration, patient age at first gluten introduction and at the onset of malabsorption symptoms. The difference between these two ages (free interval) was counted in days.

FAMILY STUDIES

Eighty seven parents and 42 healthy siblings were analysed to establish the probands' HLA genotype. A larger sample of 162 patients and 96 healthy siblings, including the HLA typed individuals, was

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considered in order to compare exposure to environmental factors in affected and healthy siblings.

CONTROLS

A control sample of 430 healthy adults from the same population was used for HLA-A,B,C specificities. The controls for HLA-DR, for Bf and for GLO were 125, 618, and 234 respectively.

HLA typing was performed with standard lymphocytotoxicity techniques. The enriched B cell suspension was used for DR, and for A,B,C the T cell fraction recovered from E rosettes. The set of DR alloantisera used permitted definition of DR1-DRw8; recognition of DRw6 is questionable, however, particularly in the presence of DR3.¹⁴ HLA-D typing was performed in mixed lymphocyte cultures with homozygous typing cells or coeliac disease patient cells as stimulators (5×10^4 /u-bottom well, 2500 rad, x-radiated) together with responder fresh lymphocytes (5×10^4 /well). Typing responses were assigned after double normalisation.¹⁵

Properdin B factor polymorphism (Bf) was studied in serum samples by high voltage agarose electrophoresis, after immunofixation with goat anti-human Bf antiserum (Atlantic Antibodies, Scarborough, ME, USA).

Glioxalase I (GLO) typing was performed on fresh haemolysate or after storage at -60°C , following standard techniques.¹⁶

Jejunal biopsies were evaluated in histological sections after the criteria of Choudhury *et al.*¹⁷ The number of intraepithelial lymphocytes (IEL) in the jejunal mucosa was evaluated according to Ferguson and Murray,¹³ and expressed $\times 1000$ epithelial cells. Concentrations of IgG, IgA, and IgM classes of serum were measured by radial immunodiffusion¹⁸ during the three jejunal biopsies.

Data evaluation The strength of association with coeliac disease was estimated for each specificity, as well as for DR genotypes, using Woolf's relative risk (RR), with Haldane's modification whenever one of

Table 1 Significant associations between HLA markers and coeliac disease

Specificity	Patients* %	Controls† %	RR	95% confidence limits	p
A9	20.0	33.7	0.5	(0.3-0.8)	$<10^{-2}$
Aw30	26.0	7.0	4.7	(2.6-8.4)	$<10^{-6}$
B8	32.0	13.7	3.0	(1.8-4.9)	$<10^{-4}$
B12	32.0	19.5	1.9	(1.2-3.4)	10^{-2}
B13	18.0	5.6	3.7	(1.9-7.2)	$<10^{-3}$
B18	22.0	13.3	1.9	(1.1-3.2)	3×10^{-2}
Bw35	10.0	30.5	0.3	(0.1-0.5)	$<10^{-5}$
Cw4	19.0	32.8	0.5	(0.3-0.8)	$<10^{-2}$
Cw6	23.5	12.8	2.1	(1.2-3.6)	10^{-2}
DR1	2.0	16.8	0.1	(0.0-0.4)	$<10^{-3}$
DR2	4.0	22.4	0.1	(0.1-0.4)	$<10^{-4}$
DR3	68.0	16.0	11.2	(5.9-21.1)	$<10^{-14}$
DRw6	4.0	17.6	0.2	(0.1-0.6)	10^{-3}
DR7	59.0	28.0	3.7	(2.1-6.5)	$<10^{-5}$
BfFl	16.0	3.6	5.2	(2.6-10.2)	$<10^{-4}$

* N=100.
† N=430 for HLA-A,B,C; 125 for HLA-DR; 618 for Bf.
RR=relative risk.

the compared values was nil.¹⁹ The significance of association was evaluated using Fisher's exact test and by doubling the computed value. Log linear analysis of three way contingency tables was done on an IBM 360 computer with BMDP Program 3F.²⁰

One locus dominant and recessive models were tested according to Thomson and Bodmer,²¹ using the frequencies of homozygous and heterozygous patient genotypes.

Results

R1 ASSOCIATION WITH MARKERS OF THE HLA REGION (Table 1)

HLA-A,B,C: significant positive associations were found for Aw30, B8, and B13. The specificities B12, B18, and Cw6 were also increased, but not signifi-

Tables 2a and 2b Expected and observed distribution for DR3-B8 and DR3-BfFl

	2a DR3-B8				2b DR3-BfFl			
	++	+-	-+	--	++	+-	-+	--
Observed								
Patients	32	36	0	32	16	52	0	32
Controls	9	10	6	98	3	11	0	70
Log linear analysis:								
Models fitted	χ^2	DF	p		χ^2	DF	p	
A - $I_{1,2} + I_{1,3} + I_{2,3}$	0.95	1	0.32		0.15	1	0.70	
B - $I_{1,3} + I_{2,3}$	55.51	2	$<10^{-12}$		41.98	2	$<10^{-9}$	
C - $I_{1,2} + I_{2,3}$	1.08	2	0.58		0.21	2	0.90	
D - $I_{1,2} + I_{1,3}$	46.86	2	$<10^{-10}$		21.86	2	$<10^{-4}$	

1=Coeliac disease; 2=HLA-DR3; 3=HLA-B8 or BfFl.

cantly so after correction for the number of comparisons ($p \geq 10^{-2}$).

HLA-DR: the strongest and most significant associations were for DR3, which was present in 68% of the patients against 16% in the controls, and DR7 (59% and 28%). This agrees with previous findings⁸ and those of other workers.^{7, 22}

Significant negative associations ($p < 10^{-2}$) were found for A9; Bw35, Cw4, DR1, DR2, and DRw6 as previously reported in a smaller sample⁸ and in other populations.²²

Properdin B factor: a significant association was only found for BfI, which is known to be associated with DR3 (16% vs 3.6%). A slight but not significant increase was also noted for BfF (in Italians, this is associated with DR7 (F Malavasi, unpublished)).

GLO displayed no difference from controls.

R2 MULTIWAY CONTINGENCY TABLES

The following approach was used because many of these specificities are interassociated by linkage disequilibrium. The observed distribution of two markers of different loci – for example, DR3 and B8 or BfI – in patients and controls was compared with those expected from several models for complex interaction. First, the 'complete' model was analysed. This assumes all possible interactions $I_{1,2,3}$ (I = disease vs control, 2 = DR3 present vs absent, 3 = B8 or BfI present vs absent) + $I_{1,2} + I_{1,3} + I_{2,3}$. Then simpler models omitting one of these I 's sequentially were examined. The expected and observed distributions are shown in Table 2a for DR3-B8 and 2b for DR3-BfI. In both cases model C, based on the association between DR3 and disease + the linkage disequilibrium between DR3 and B8 or BfI respectively, showed a good fit with the observed data. Interactions $I_{1,3}$ (direct association between disease and B8 or BfI) and $I_{1,2,3}$ (the so-called 'haplotype effect'²³) thus proved to be unnecessary, although they were not excluded (as shown by the good fit of model A). For the sake of simplicity, therefore, our analysis will be confined to the HLA-DR locus.

R3 HLA-D/DR GENOTYPES

DR typing alone classified patients and controls as positive for either DR3 or DR7, positive for DR3 and DR7, or negative for both antigens. Furthermore, the D/DR genotypes of 16 patients who carried DR3 but not a second DR allele (apparent DR3 homozygotes) were determined through family studies and by using their lymphocytes as stimulators in MLC against a panel of HLA-D typed individuals. Nine were D/DR homozygous according to both criteria, while seven were hetero-

Fig. 1 Relative risk (RR) of different HLA-DR genotypes with 95% confidence limits. Absolute numbers of patients and controls are indicated in bottom line. Genotype 7/X comprises three 7/7 homozygous patients (see text).

zygous (two DR3/DRw6, two DR3/Dw9, and three DR3/blank). In the control sample, only one out of three apparent homozygotes was D/DR homozygous, in agreement with Hardy-Weinberg expectations (gene frequency 0.0835, expected homozygotes 0.87, $\chi^2=0.02$).

The genotype frequencies of patients and controls were then compared using Woolf's formula (Fig. 1): genotypes 3/3 and 3/7 displayed high risks (12.2 and 8.5 respectively), whereas lower risks were found for the 3/X (RR = 3.5) and 7/X (RR = 1.5) genotypes (X = any allele other than 3 and 7). Among the 7/X, three apparent DR7 homozygotes were found (expected 2.1, not significant). The X/X genotype showed a risk close to nil, as it was only represented by three patients, all of whom carried the DR4 allele.

R4 SEX

The overall sex ratio was 0.64, in agreement with other paediatric samples. Equal numbers of males and females were found in the high risk DR genotypes 3/3 and 3/7 (M/F = 1.05), while females were in excess in the low risk group 3/X (M/F = 0.70) and even more so in the 7/X group (M/F = 0.26, $p < 0.05$) (Table 3).

R5 HETEROGENEITY BETWEEN DIFFERENT HLA-DR GENOTYPES

ENVIRONMENTAL FACTORS

Significantly fewer patients were breast fed (at least one month) compared with unrelated controls (50% vs 82.5%, RR = 2.8, $p < 10^{-3}$), but not with healthy siblings (52.8%). Early gluten introduction (<3 months of age) was more frequent in patients (48.5%) than in unrelated controls (17.5%, RR = 4.5, $p < 10^{-5}$) and in healthy siblings (26.2%, RR = 2.7, $p < 10^{-3}$).

CLINICAL PROGRESSION

(i) The length of time between first gluten introduction and appearance of clinical symptoms (free interval) was longer in the low risk group 7/X (233 ± 296 days) than in the remaining DR3 positive genotypes (142.5 ± 74 days, $p < 0.05$), while these did not differ among themselves. (ii) In 36 patients the clinical symptoms at onset were atypical for coeliac disease (10 cases showed constipation, nine isolated failure to thrive, five dwarfism, four anorexia, four vomiting, two muscular hypotonia, one oedema, one intolerance to milk proteins). In boys this atypical onset was more common in the low risk 3/X and 7/X genotypes than in the high risk genotypes (Table 3), while in girls it was not related to DR.

Table 3 Relationship between atypical symptoms at onset, sex and DR genotypes

HLA-DR	3/3+3/7		3/X+7/X		χ^2	p
	(No)	Atypical %	(No)	Atypical %		
Males	20	20.0	18	61.1	6.7	$< 2 \times 10^{-2}$
Females	19	31.6	40	35.0	0.07	NS

HISTOLOGICAL EVALUATION OF JEJUNAL BIOPSIES
In all patients, the mucosal lesion fulfilled the criteria required for diagnosis.¹¹ Quantitative evaluation of the histological sections was only possible in some cases (Fig. 2). Mean IEL number was higher in the first biopsy, lower in the second on gluten free diet, and rose in the third biopsy after gluten challenge. Comparison with the DR genotype showed that the IEL number was higher in DR3 positive patients in the third biopsy, irrespective of sex and age, while there was no significant difference in the first and second biopsies.

SERUM IMMUNOGLOBULIN CONCENTRATIONS
IgG and IgM were much the same in the three samples. IgM were significantly higher in girls than in boys (1.3 mg/ml vs 1.03 mg/ml, $p < 0.05$), irrespective of the HLA genotype.

Selective IgA deficiency was present in five patients, against a frequency of 1/800 in the Italian population ($RR = 42$, $p < 10^{-6}$) (unpublished

Fig. 2 Intraepithelial lymphocytes (IEL) counts of DR3-positive and DR3-negative patients. One standard deviation above and below mean is indicated.

results). All were DR3 positive and belonged to the high risk genotypes.

In the remaining patients, IgA concentrations showed significant differences between disease stage, age, and DR genotype (Table 4): (i) IgA rose during the active stages of coeliac disease (first and third sample), and was lower during remission on gluten free diet. In later follow-up, patients who did not strictly adhere to the prescribed gluten free diet showed an increase of IgA concentrations, even in the absence of overt clinical relapse (N Ansaldi, unpublished). (ii) In the second sample, IgA concentrations were significantly lower in the DR3 positive patients than in the DR3 negatives. In contrast, no correlation with DR was found in the first sample at the onset of malabsorption, nor in the third sample after gluten challenge.

Discussion

The genetics of coeliac disease are probably multifactorial, as shown by classic studies in populations and families,^{24 25} by the wide variety of the clinical features,¹² and by the existence of asymptomatic

Table 4 Serum IgA concentrations in coeliac disease

DR3	1st biopsy				2nd biopsy				3rd biopsy			
	No	mg/ml	SD	p	No	mg/ml	SD	p	No	mg/ml	SD	p
+	46	1.38	0.67	NS	46	0.82	0.56	<0.01	42	1.10	0.44	NS
-	25	1.68	1.38		18	1.25	0.94		14	1.26	0.60	

individuals who do not reach the full expression required for diagnosis.^{2,26} Association with genetic markers has clarified some of the genes involved, but has failed to provide a simple genetic model for susceptibility.¹⁰ More than one HLA-linked gene and HLA-independent genes have been postulated.⁹ Our data point to involvement of HLA and sex in the onset and progress of coeliac disease, and suggest that its severity is to some extent correlated with the weight of genetic factors.

ASSOCIATION WITH HLA

Several HLA specificities are involved with different strengths. This complex pattern can be attributed to (i) the association of coeliac disease with two DR alleles, DR3 and DR7, and (ii) linkage disequilibrium between these factors and alleles of nearby loci.

Linkage disequilibrium may account for the increase of Aw30, B18, B8, BfF1, which are associated with DR3 in the general population, as most patients who are positive for these specificities belong to the DR3 positive groups. Similarly, the increase of B12, B13, Cw6, and BfF is probably because of their linkage disequilibrium with DR7. This simple explanation has been criticised by Thomson and Bodmer,²³ who stress the need for quantitative analysis to rule out the so-called 'haplotype effect' – that is, the increased risk of particular haplotypes that may preferentially carry the 'disease gene'. In the same way, Albert *et al.*²² have noted that the involvement of B8 may vary between different DR3 associated diseases, suggesting that the B and, by extension, the Bf loci may contribute to susceptibility to coeliac disease. Our approach with the log linear analysis of multi-way contingency tables does not show, but does not rule out, either a direct association between B8 and coeliac disease, or an haplotype effect. An answer to the question probably requires *in vitro* models able to dissect the mechanism of immune damage, and classify the role of class I, II, and III gene products in different stages of the disease.²⁷

The dual association with DR3 and DR7 still represents the most stimulating result of HLA studies in coeliac disease. The original findings by Albert *et al.*,⁶ Betuel *et al.*,⁷ and ourselves⁸ have been substantiated in several populations.²⁸ The strength of association is higher for DR3 in northern countries, while that of DR7 is generally lower and limited to southern countries. In the present data, the different weight of DR3 and DR7 is supported by several findings: (i) DR3 is more strongly associated than DR7; (ii) only DR3 shows a gene-dose effect – that is, genotype 3/3 has a higher risk than 3/X; (iii) the contribution of DR7 is only

evident in the presence of DR3, where it increases the risk of DR3 heterozygotes up to the level of DR3/3 homozygotes. The contribution of DR7 alone (7/7 and 7/X) is weaker, and not even statistically significant in the usual Woolf test; nevertheless, some risk can also be attributed to these genotypes when compared with X/X (Svejgaard and Ryder²⁹); in fact they account for almost one-third of the patients.

The association with two DR alleles resembles that found in type I diabetes, which is associated with DR3 and DR4.^{29,30} Two genetic hypotheses can explain these findings. The first assumes an illness gene *d*, in linkage disequilibrium with both DR3 and DR7; a tentative candidate might be the supertypic specificity MB2, the product of a separate gene, distinct from the classic DR locus.³¹ If *d* is the presence of either DR3 or DR7, and *D* the absence of both alleles, there are three possible models which differ with regard to the penetrance of the *dd* (*p*₁) and *Dd* (*p*₂) genotypes:³² a dominant model with *p*₁ = *p*₂; a recessive model with *p*₂ = 0, and an intermediate model with *p*₁ = *p*₂. Following Thomson and Bodmer's procedure,²¹ the expected genotype frequencies were calculated for the first two models; both were rejected, the dominant for an excess of *dd* and a defect of *Dd*, and the recessive for a defect of *dd* and an excess of *Dd* (Table 5). The intermediate model, though preferable, is quantitatively undetermined, as actual estimates of *p*₁ and *p*₂ are lacking. Appropriate theoretical penetrance and gene frequency values can always be found to fit the genotype proportions observed.

Alternatively, in the second hypothesis, the DR specificities are not just markers associated with an 'illness gene', but molecules directly involved in the pathogenetic mechanism. Experimental data on the role of Ia-like products in cell-to-cell recognition and the regulation of immune response support this view.³³ In this case, genetic analysis of susceptibility is simplified, as the susceptible genotypes are directly available; on the other hand, it remains to

Table 5 Test of the genetic model assuming a single gene *d* associated with DR3 and DR7

Genotype	Observed	Expected Dominant	χ^2	Expected Recessive	χ^2
3/3 } <i>d/d</i>	42	22.3	17.4	68.4	10.2
3/7 } <i>d/d</i>					
7/7 } <i>d/d</i>					
3/X } <i>D/d</i>	55	74.7	5.2	28.6	24.4
7/X } <i>D/d</i>					
X/X } <i>D/D</i>	3	3	0	3	0
Total	100	100	22.6	100	34.6

be explained why the same DR product, say DR3, is associated with a variety of diseases, and why these only appear in a limited proportion of individuals with the relevant DR antigen. The second hypothesis thus requires the identification of additional genetic or environmental risk factors.

SEX

Our data indicate that coeliac disease is more common in females. As both sexes were equally exposed to the known environmental risk factors – that is, artificial feeding and early introduction of gluten – the difference may be attributed to a lower genetic threshold in females. This view is further supported when sex and HLA genotypes are considered jointly. In our sample, the M/F ratio progressively decreases from the high risk 3/3 and 3/7 to the low risk DR genotype 3/X, and even more to the 7/X. The simplest interpretation of this trend is that the penetrance of the low risk HLA genotypes is increased in the context of the susceptible female 'background'. A similar observation has been reported by Batchelor *et al* in drug induced SLE,³⁴ where the female sex again acted as a risk factor, together with HLA-DR4 and low-acetylator phenotype.

PHENOTYPIC VARIABILITY

The significance of this complex genetic pattern can be evaluated by comparison with the well known variability of the clinical features of coeliac disease. The findings that most suggested a correlation with the weight of genetic factors were the longer free interval in the low risk DR7/X genotype, and the proportion of cases with atypical onset; this was more frequent in male DR3/X and 7/X patients, perhaps because a low risk conferred by both DR and sex can only induce coeliac disease when additional precipitating processes cause a non-specific mucosal lesion and trigger the development of gluten sensitivity. Subjective evidence of this kind may be biased by uncontrolled environmental factors and inaccurate clinical records. These findings, therefore, require further substantiation.

The suggestion, however, that patients with lower HLA-dependent risk may run a milder coeliac disease course is also supported by the laboratory and immunological data. In this respect the most significant finding is the high number of IEL in DR3 positive patients after gluten challenge; this may be responsible for the early appearance of their symptoms. These patients may have higher cell mediated reactivity against jejunal mucosa in the presence of gluten, as previously shown by Falchuck *et al*,²⁷ who found more severe tissue damage in patients carrying HLA-B8 than in patients negative

for this antigen, and by Cunningham-Rundles *et al*,³⁵ who showed a higher *in vitro* response to gluten of peripheral lymphocytes from normal individuals positive for HLA-B8 or DR3.

Although variations in Ig concentrations have been described in coeliac disease, mainly for IgA and IgG,¹ the involvement of humoral immunity in its pathogenesis is less understood. The IgA system is obviously a candidate because of its role against foreign antigens at the mucosal barrier. The presence of five patients in our series with selective IgA deficiency confirms that susceptibility to coeliac disease may occasionally be due to an impaired barrier to gluten. A second, but not alternative, explanation is that IgA deficiency and coeliac disease derive from a common autoimmune mechanism, as suggested by their association with DR3, and by the increased risk in IgA deficiency of other DR3 associated diseases, such as type I diabetes,³⁶ dermatitis herpetiformis, and SLE.³⁷

In the remaining patients, who are not deficient, serum concentrations of IgA increased during the active stages – that is, at the first and third biopsy. In contrast, the second sample probably came close to the individual 'normal' value. It is tempting to attribute the lower IgA concentrations of the DR3 positive patients to some mechanism in common with selective IgA deficiency, with impaired local defence in both cases. Estimation of pathological changes in IgA levels, however, is complicated by genetically controlled variations observed in the general population, and also by the physiological increase from birth until adolescence.³⁸ Our findings therefore require substantiation from longitudinal studies of IgA levels on patients, healthy siblings, and unrelated controls of known HLA genotype.

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