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# Novel HLA Class II-associated structural patterns in coeliac disease and type I diabetes

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## SUMMARY

Cell membrane antigens were precipitated from EBV transformed cell lines by a monomorphic DR monoclonal antibody. Three mutually exclusive patterns with two glycoproteins (g25 and g28) that had not been previously identified, were observed. The first, $g25^+/g28^-$ was found in all cell lines from 40 healthy individuals; a second, $g25^-/g28^-$ was found in 4/7 coeliac and 2/4 IDDM patients and a third, $g25^+/g28^+$ was found in 3/7 coeliac and 1/4 IDDM patients. RFLP analysis with Class II alpha and beta chain probes and several restriction enzymes did not correlate with either of the disease associated patterns. Several possibilities regarding the identity and mode of action of the two polypeptides are described.

**Keywords** HLA-DR 2-D gel coeliac disease IDDM

## INTRODUCTION

Gluten sensitive enteropathy or coeliac disease (CD) is associated with the serologically defined HLA Class II antigens DR3 and DR7, whereas insulin dependent (Type I) diabetes mellitus (IDDM) is associated with DR3 and DR4. The highest relative risk for both conditions is given by the heterozygous presence of DR3 together with DR7 in CD and DR4 in IDDM. Southern blot studies using the HLA-DQ alpha and beta probes in IDDM patients with the DR4 haplotype and CD patients with the DR7 haplotype have revealed that the DQ beta and DX alpha restriction fragment length polymorphism (RFLP) more closely associate with disease susceptibility than the corresponding DR antigens (Owerbach *et al.*, 1983; Cohen-Haguenauer *et al.*, 1985; Hitman *et al.*, 1986; Festenstein *et al.*, 1986; Nepom *et al.*, 1986; Howell *et al.*, 1987). However, Stetler, Grumet & Erlich (1985) have identified two subtypes of DR3 based on a DR alpha polymorphism one of which is increased in patients with IDDM. In our extended data there was no difference in their distribution in IDDM and CD patients and controls (Hitman *et al.*, 1987). Although we could demonstrate DR3-related preferential allelic associations in both conditions extending from DR alpha through to DQ and DX alpha (Hitman *et al.*, 1987; Gorski *et al.*, 1987), none of the individual alleles correlated more closely than another. The DR3 related susceptibility gene thus remains elusive and may indeed be located within the DR subregion.

We are now using a different approach for identifying HLA associated susceptibility genes by looking for structural protein changes in Class II associated gene products. The rationale behind this approach is that putative aberrant proteins may be responsible for susceptibility to CD or IDDM and enable the controlling genes to be identified through sequencing the proteins. We now report on molecular patterns obtained after SDS-PAGE 2-dimensional (2-D) analysis of EBV transformed lymphoblastoid cell lines (LCL) membrane antigens precipitated by the well-characterized DR monoclonal antibody L243.

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## MATERIALS AND METHODS

*Patients*

Eleven unrelated Caucasoid patients (seven CD and four IDDM) were selected from the Outpatients clinics of The Queen Elizabeth Hospital, Hackney and The London Hospital respectively. The diagnosis of CD and IDDM was based on previously described criteria (Hitman *et al.*, 1987).

*Lymphoblastoid cell lines*

The HLA Class II phenotypes of the patients from which cell lines were established by standard Epstein–Barr virus (EBV) transformation are shown in Table 1. The panel of LCLs from healthy controls ($n=40$) covered most DR and Dw specificities but particularly DR3 (AS, 127 and WT49), DR4 (Fle, MCF, BM14, JHA) and DR7 (SHA, LBU and JMF).

**Table 1.** 2-D gel patterns of novel g25 and g28 molecules precipitated by a DR monoclonal antibody from LCLs of coeliac and IDDM patients

| Cells† | Diagnosis | HLA-DR | HLA-DQ | 2-D gel | pattern* |
|---|---|---|---|---|---|
| HOM-2 | HTC | DR1/1 | DQw1/1 | g25 | — |
| DMg | Coeliac | DR3 | DQw2 | g25 | g28 |
| JGr | Coeliac | DR3/11 | DQw2/3 | g25 | g28 |
| DPa | Coeliac | DR3/13 | DQw1/2 | — | — |
| LDa | Coeliac | DR3/7 | DQw2 | — | — |
| DMi | Coeliac | DR3/7 | DQw2 | — | — |
| SBR | Coeliac | DR3/7 | DQw2/3 | g25 | g28 |
| MHac | Coeliac | DR4/4 | DQw3/3 | — | — |
| Mrem | IDDM | DR1/3 | DQw1/2 | g25 | — |
| SAJ | IDDM | DR3/7 | DQw2 | — | — |
| SSm | IDDM | DR4 | DQw3 | g25 | g28 |
| Lay | IDDM | DR4/13 | DQw1/3 | — | — |

* 4 h radio-labelling and neuraminidase treatment.
† Local designation of EBV transformed cell.
HTC, homologous typing cell.

*Biosynthetic labelling and preparation of soluble antigens*
Lymphoblastoid cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (Flow laboratories, UK). $20 \times 10^6$ cells were harvested at exponential phase of growth, centrifuged (86 *g* for 15 min at 37°C) and washed once in PBS. The cells were then resuspended in 4 ml of MEM without L-methionine (Gibco Limited, UK) supplemented with 1% non-dialysed FCS and cultured for 1 h at 37°C in a 5% $CO_2$ atmosphere. Then, $^{35}$S-Met (Amersham International, UK) was added (0·5 mCi = 185 MBq) and the cells were further incubated for 4 h. The radiolabelling was terminated by washing the cells three times with PBS (4°C) supplemented with 0·02% sodium azide and 1 mg/ml L-Met. Finally, the $^{35}$S-Met labelled cells were resuspended in 2 ml NP-40 lysis buffer (10 mM Tris-HCl at pH 7·4, 150 mM NaCl, 0·5% NP-40, 0·02% $NaN_3$ and 1·0 mM PMSF) and incubated for 30 min at 4°C. The insoluble material was removed by centrifugation at 10,000 *g* for 30 min. The supernatants containing soluble antigen extracts were collected and stored at −20°C.

*Immunoprecipitation experiments and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*
The labelled glycoproteins were 'precleared' successively with 100 μl protein-A bearing *Staphylococcus aureus* Cowan I (SACI), 100 μl of SACI coupled with rabbit anti-mouse and 100 μl of SACI coupled with anti-human immunoglobulin. Then, samples ($2 \times 10^6$ cell equivalents) of the glycoproteins were incubated with an excess of the L243 MoAb (used as purified or undiluted ascites fluid) at 4°C for 1 h. The antigen–antibody precipitates were incubated with 30 μl SACI, extensively washed in lysis buffer with or without NP-40 and stored at −20°C.

*Two-dimensional gel electrophoresis*
The precipitated material was eluted from the SACI pellets by incubation in non-equilibrium pH gradient electrophoresis (NEPHGE) sample buffer (de Krester *et al.*, 1982). The eluted polypeptides were separated by NEPHGE on a 12 cm gel containing carrier ampholines (pH range 3·5 to 10·0 LKB Instruments Ltd, Croydon).

The samples were electrophoresed for a total of 2700 Vh at 200 V constant voltage. The gels were then extruded from the tube and equilibrated for 2 h in SDS-PAGE sample buffer before separation according to molecular weight.

*Neuraminidase digestion*
The eluted antigens (see above) were incubated overnight at 37°C in the presence of 0·1 M sodium acetate 0·3 M NaCl–0·2% CaCl (pH 5·5) and 25 mU of *Vibrio* cholera neuraminidase (Calbiochem).

*DNA studies*
DNA was prepared from either whole blood samples or lymphoblast cell lines by the method of Kunkel *et al.* (1977), from nine individuals; three were $g25^+/g28^+$, three were $g25^-/g28^-$ and three were $g25^+/g28^-$. The DNA was digested separately with Taq 1, Bgl II and Rsa I (BAM HI was additionally used for the DZ alpha probe), separated according to size by agarose gel electrophoresis and transferred to nylon membranes (Gene Screen Plus) by a modified method of Southern blotting (Southern, 1975). Two complete sets of membranes were prepared which serially hybridized to one of the two sets of probes: (i) DQ-beta, DR-beta and DP-beta and (ii) DR-alpha, DQ-alpha (which also identifies DR-alpha), DZ-alpha and DP-alpha. Membranes were washed down to high stringency and gene related sequences identified by autoradiography.

## RESULTS

The 2-D molecular pattern obtained after immunoprecipitation with the well-established HLA-DR specific monomorphic antibody L243 revealed an HLA-DR alpha chain and closely spaced HLA-DR beta chains (Fig. 1) from all the cells tested. The HLA-DR alpha chains focused at a single pI (5·5) and approximate molecular weight 33 kD. The HLA-DR beta chain profiles comprised a series of closely related spots of approximate pI 7·5 and molecular weight 30 kD (Fig. 1A). There were no apparent alpha or beta chain differences between the normal and the IDDM and coeliac cell lines other than a persistent variability of the DR beta profile related to their serological phenotype (data not shown).

In addition to the HLA-DR alpha and beta subunits, the invariant chain (31 kD, pI 7·5), actin (45 kD, pI 6·0) and a band which we have designated g25 (25 kD, pI 6·5) were present as indicated in Fig 1A. Even after partial de-sialation with neuraminidase treatment (Rudd *et al.*, 1984) this g25 band is invariant (Table 1). Pulse chase experiments revealed that it is expressed in parallel, with a similar rate of synthesis, as Class II heterodimers (Fig. 1D). The g25, invariant and actin bands were always present in cell lines from all 40 healthy controls.

In 6/11 patients (4/7 CD and 2/4 IDDM), the g25 polypeptide was absent (Fig. 1B, Table 2) even after prolonged exposure (15 days) of the gels. In 4/11 patients (3/7 CD and 1/4 IDDM), an additional spot (Fig. 1C, Table 2) which we have designated g28 (28 kD pI 7·5) was identified but was not apparent in any of the 40 control gels even after prolonged exposure. Neither the presence of the g28 nor the absence of the g25 spot correlated with the HLA-DR phenotypes (Table 1). In order to determine

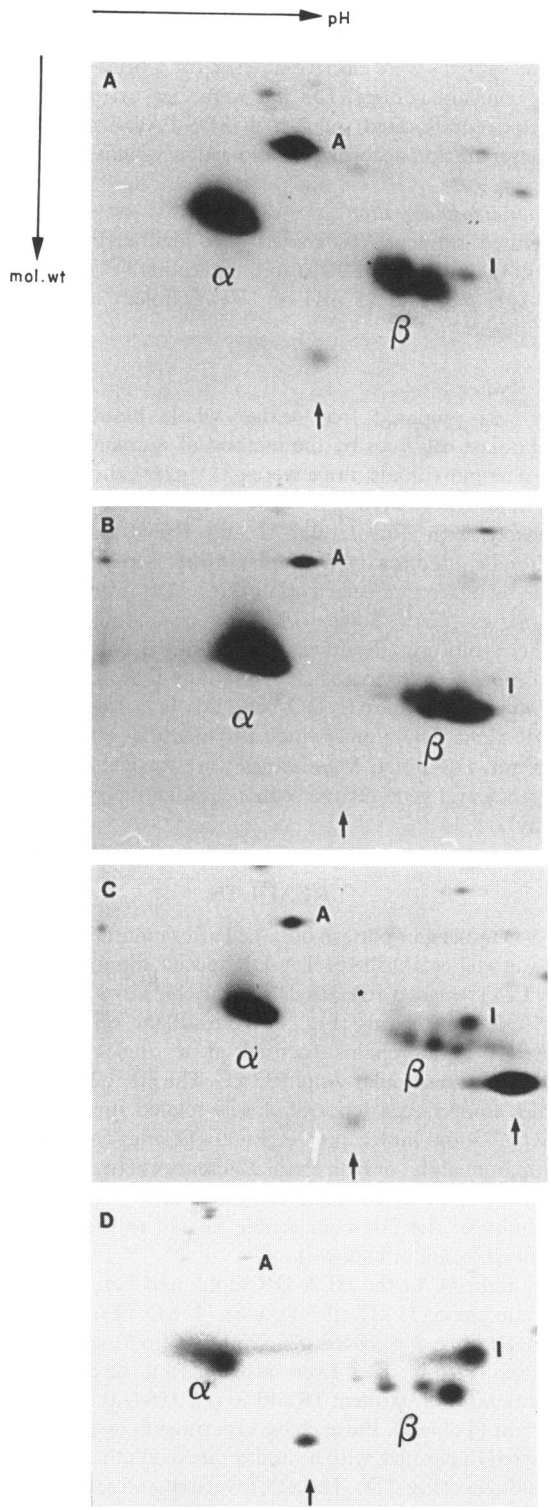


**Fig. 1.** Autoradiograph of four 2-D gel molecular patterns obtained after immunoprecipitation of membrane lysates from EBV lymphoblastoid cell lines with the DR specific monomorphic antibody L243. The 2-D gel spots corresponding to the alpha chain (α), and beta chain (β), Actin (A) and invariant chain (I) are indicated in all four panels. Panel A: The presence of the band designated g25 (vertical arrow). Panel B: The absence of the g25 band (vertical arrow). Panel C: The presence of g25 and g28 bands (vertical arrow). Panel D: The presence of g25 band after 5 minutes pulse chase radiolabelling.

**Table 2.** Presence and absence of g25 and g28 protein molecules in healthy controls and patients with coeliac disease and IDDM

| | $gp25^+$ $gp28^-$ | $gp25^-$ $gp28^-$ | $gp25^+$ $gp28^+$ |
|---|---|---|---|
| Controls ($n=40$) | 40 | 0 | 0 |
| IDDM ($n=4$) | 1 | 2 | 1 |
| CD ($n=7$) | 0 | 4 | 3 |

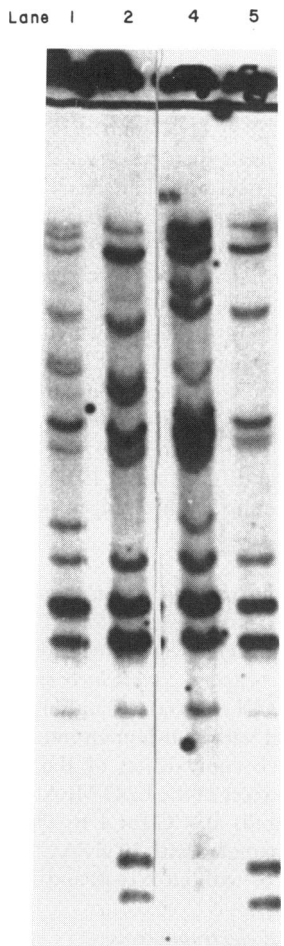


**Fig. 2.** Autoradiograph of RFLP obtained with DNA from individuals with or without g25 and g28 bands. There is no fragment common to Lanes 1 and 2 ($g25^+$, $g28^-$) which is absent from Lanes 4 and 5 ($g25^-$, $g28^+$) and *vice versa*.

whether there was a corresponding HLA-D region DNA polymorphism for these glycosylated proteins, nine individuals (three with $gp25^+/g28^+$, three with $gp25^-/g28^-$ and three with $g25^+/g28^-$) of varied DR phenotypes were selected for RFLP studies. The restriction enzymes Bgl II, Taq 1 and Rsa I revealed informative RFLPs for the DR-beta, DQ-beta, DQ-alpha and DP-beta genes; two enzymes detected RFLPs of the DR-alpha (Bgl II and Rsa I) and DP-alpha (Bgl II and Taq 1) genes and one enzyme detected RFLP of the DZ-alpha (Bam HI), DX-alpha (Taq 1) and DQ-beta (Bgl II) genes. Different RFLP patterns derived from the various Class II probes did not correspond to the presence or absence of the g28 or g25 spots (Fig. 2).

## DISCUSSION

CD and IDDM are associated with HLA susceptibility genes currently marked by HLA Class II DR and DQ serologically detected antigens, HLA-D (Dw) locus lymphocyte activating determinants defined by cellular typing and HLA Class II region RFLPs.

To identify putative products of the HLA Class II susceptibility genes we have screened DR precipitates of cell membrane glycoproteins from lymphoblastoid cells of patients with CD, IDDM and healthy controls employing 2-D gel analysis. The classical description of 2-D gel patterns obtained after precipitation with DR monoclonal antibodies comprises four components: a Class II alpha chain spot, Class II beta chain spots, the invariant chain and actin. We have now identified an additional polypeptide of approximate molecular weight 25 kD and pI 6·5 (g25) which although not previously described has been noted by other workers (R. Bontrop, pers. comm.). In addition, the g25 spot can be readily distinguished in published data (Zelisewiki *et al.*, 1987) and in the data submitted to the Xth Histocompatibility Workshop (Fernandez *et al.*, unpublished data).

There are several possibilities regarding the identity of the g25 polypeptide. It may represent a non-HLA product non-covalently complexed to the DR alpha and beta heterodimers similar to those polypeptides reported to co-precipitate with H-2 I-E molecules (Monaco & McDevitt, 1984; Zecher *et al.*, 1984). Alternatively, this protein could belong to the family of HLA Class II antigens not yet identified, such as a secreted Class II molecule lacking the transmembrane segment (R. Inoko, pers. comm.) thus having a lower molecular weight. It is an attractive possibility that g25 is related to tumour necrosis factor B (lymphotoxin) which has a similar molecular weight (20–25 kD), is a product of the non-Class II region MHC (Spies *et al.*, 1986) and is known to influence HLA levels of cultured cells. The absence of g25 is 4/11 CD and 2/4 IDDM patients is intriguing. Until the identity of the spot is established, we cannot determine whether failure to express the product is involved in the cause of or is consequent on the disease processes.

A second novel polypeptide g28 was observed in three patients with CD and 1 patient with IDDM and was absent in all 40 controls including the five LCLs used in the Xth Histocompatibility Workshop. It can be distinguished from the DR beta chains by its lower molecular weight and more basic pI. The genetic control of the polypeptide is not known. The absence of a corresponding RFLP after probing with the known HLA Class II genes does not exclude the possibility that the polypeptide might be within the HLA Class II region. If it is a variant of an existing or another Class II beta chain, we speculate that it is coded by a susceptibility gene in association with the DR3 haplotype as DR3 is common to both diseases whereas the DR4-associated gene in IDDM and the DR7-related gene in CD are closer to the DQ subregion. Whether the gene controlling g28 is carried on the same haplotype as the unexpressed g25 gene cannot be determined without studies on informative families.

Our preliminary results thus indicate that two molecular patterns can be obtained after precipitation with a well-defined DR MoAb from CD and IDDM patients that differ from the control group. Further studies directed at determining the sequence of these glycoproteins with the eventual aim of cloning the corresponding genes are in progress.

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