

HLA-D gene studies in relation to immune responsiveness to a grass allergen *Lol p III*

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Abstract. The grass pollen allergen *Lol p III* (M_r 11 000) is a well-characterized antigen that has been found useful in immunogenetic studies of human immune responsiveness. Since immune responsiveness to this allergen is associated with HLA-DR3, we investigated whether there was any sequence in the *HLA-D* region that would render a person “susceptible” [antibody (Ab)-positive] to the allergen. By sequence-specific oligonucleotide (SSO) slot-blot and sequence analyses of polymerase chain reaction (PCR)-amplified genomic DNA from *Lol p III* responder and nonresponder subjects, Ab responsiveness was found to be strongly associated with the sequence Glu-Tyr-Ser-Thr-Ser (EYSTS), present in the first polymorphic regions of DR β I polypeptide chains of DR3, DR11 (split of DR5), and DRw6. Of the 41 grass-allergic subjects investigated, 19 had the EYSTS sequence, of whom 18 (95%) were *Lol p III* immunoglobulin G (IgG) Ab responders; among the 22 EYSTS[−] subjects, ten were *Lol p III* responders ($P=0.001$, relative risk=21.6). No such association was found with any polymorphic sequences in other DR β chains, or in DQ α I and DQ β I chains. These findings suggest that the EYSTS sequence is important in the presentation of an epitope of *Lol p III*; other sequence(s) may be involved in the presentation of other epitope(s). To our knowledge, this is the first demonstration of a strong association between a specific *HLA* sequence and immune responsiveness to a well-defined antigen. The paper shows that presence of the EYSTS sequence classifies subjects as *Lol p III* responders in 18/19 cases.

disorders (Marsh et al. 1982a, b; Goodfriend et al. 1985; Tiwari and Terasaki 1985; Freidhoff et al. 1988; Ansari et al. 1989a, b). The relationship between *HLA* sequences and specific immune disorders has been the subject of some very important recent publications (Todd et al. 1987, 1988; Horn et al. 1988; Scharf et al. 1988; Ronningen et al. 1989a, b; Sheehy et al. 1989; Yamagata et al. 1989; Awata et al. 1990; Winchester 1990; Khalil et al. 1990). However, similar studies have not been performed with clearly identified and well-defined antigens. In the present study, we investigated the *HLA-D* gene sequence that is associated with the immune responsiveness to a well-defined allergen, *Lol p III* from ryegrass pollen.

Grass pollen allergy is a major human health problem throughout the world, perennial ryegrass (*Lolium perenne*) being one of the more important species causing such allergy. Human immunoglobulin E (IgE) antibody (Ab) and IgG Ab responses to a grass allergen, *Lol p III* (M_r 11 000) have been found to be associated with HLA-DR3 (Ansari et al. 1989a, b). We investigated whether there was any sequence in the *HLA-D* region that would render a person “responder” (Ab-positive) to the allergen. This was carried out by sequence-specific oligonucleotide (SSO) slot-blot and sequence analyses of polymerase chain reaction (PCR)-amplified genomic DNA from *Lol p III* responder and nonresponder subjects who had all received immunotherapy (Rx) with grass pollen extract. Our present report represents the first demonstration of a strong association between a specific *HLA-D* sequence and immune responsiveness to a well-defined antigen.

Introduction

The *HLA* gene complex contributes to the genetic susceptibility of individuals to various allergies and autoimmune

Materials and methods

Study subjects. The study subjects included 41 grass allergic patients who had all received Rx with mixed grass pollen extracts in our allergy clinic. These subjects were sampled from a group of 67 Caucasoid patients who had been used in previous studies (Freidhoff et al. 1988; Ansari et al. 1989b) and had been typed for HLA-DR and -DQ by standard

serological procedures (van Rood et al. 1975; Bias et al. 1981). *Lol p* III-specific IgG Ab levels both pre- and post-Rx were available on all the 41 patients.

Homozygous typing cells. Seven homozygous typing cell lines (HTCs) were used as controls. The cell lines NOL (DR2/Dw2), HAR (DRw17/Dw3), ATH (DRw11a/Dw5), APD (DRw13/Dw18), and OH (DRw14/Dw9) were kindly provided by A. Termijtelen (University Hospital, Leiden, The Netherlands); SS (DR4/Dw13) and BS (DR4/Dw4) were from W. B. Bias (The Johns Hopkins University, Baltimore, Maryland).

DNA purification. Genomic DNA was purified from patients' peripheral blood mononuclear cells or from the HTCs as described before (Zwollo et al. 1989).

PCR amplifications. The second-exon gene segments of *DRB*, *DQA1*, and *DQB1* were amplified using the primer sets shown in Figure 1. The primer set PZ5A2 and PZ2A (Zwollo et al. 1990) consistently amplifies the following genes efficiently: *DRB5*, *DRB3* pseudogene, and all *DRB1* genes except the DR2-associated *DRB1* genes, namely *DRB1*1501*, *1502*, *1601*, and *1602*. In addition, this primer set also amplifies less efficiently and to varying degrees the *DRB4* genes, *DRB3* expressed genes (*DRw52*), and those *DRB1* genes that are DR2-associated, namely *DRB1*1501*, *1502*, *1601*, and *1602*. The *DRB2* pseudogene is not amplified by this primer set. We designed another upstream primer, A52PR, which in conjunction with the downstream primer PZ2A amplifies only *DRB3*. The *DQA* primer set GH26 and GH27 is from Scharf and co-workers (1986). The primers DGM4 and DGM7B were used to amplify *DQB* second exons (Zwollo et al. 1990). All PCRs were performed using *Taq* polymerase. Typically, a reaction volume of 105 μ l contained: 1 μ g genomic DNA, 100 pmol of each of the primers, 75 nmol of each of all four dNTPs in PCR buffer (25 mM KCl, 10 mM Tris-HCl at pH 8.4, 6 mM MgCl₂, 0.01% gelatin), and 2.5 units *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Connecticut). It should be noted that the stock *Taq* polymerase from Perkin Elmer Cetus was 5

units/ μ l and contained 0.1 mM ethylenediaminetetraacetate (EDTA); before use, it was diluted 1:1 in Tris-EDTA buffer which contained 1 mM EDTA, and then 1 μ l of the diluted enzyme (2.5 units) was added to the reaction mixture; thus, the reaction volume of 105 μ l contained 5.2 μ M EDTA, which would reduce the concentration of free Mg⁺⁺ somewhat. For all amplifications, the DNA samples were initially denatured for 10 min at 94 °C before the *Taq* polymerase was added. Further information on denaturation, annealing, extension times, and temperatures for each set of PCRs is given in Figure 1. The degree of each amplification was monitored by agarose gel electrophoresis and by the use of framework SSOs (see Results and Discussion).

Slot-blot analysis. Approximately 5% of the PCR-amplified DNA (5 μ l) was diluted to 50 μ l with water and denatured by heating to 95 °C for 5 min, immediately followed by chilling on ice. The sample was then diluted with 20 $\times$ standard sodium citrate and applied to a nylon filter (Hybond-N) using a slot-blot apparatus (BRL, Gaithersburg, Maryland). Then the filter was soaked for 5 min in a denaturing solution (1.5 M NaCl, 0.5 M NaOH) and for 1 min in a neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, 0.001 M EDTA). The filter was prehybridized in a 5 ml mixture containing 5 $\times$ saline-sodium phosphate-EDTA (SSPE; 0.75 M NaCl, 50 mM NaH₂PO₄, and 6.25 mM EDTA, pH 7.0), 5 $\times$ Denhardt's solution, and 0.5% sodium dodecyl sulfate (SDS) for 2 h at (T_d-3) °C, where T_d=[4 $\times$ $\sum$ (C+G)+2 $\times$ $\sum$ (A+T)] °C is the theoretical temperature at which 50% of the SSO is dissociated from the homologous sequence in a 1 M NaCl solution (Suggs et al. 1981). The filter was incubated with ³²P-labeled SSO (30 ng) in the prehybridization mixture for 1 h at (T_d-3) °C. The filter was washed twice for 15 min at 24 °C in 2 $\times$ SSPE containing 0.1% SDS, followed by 10-min successive washings in 6 $\times$ SSPE containing 0.1% SDS at 2 °C intervals starting at (T_d-4) °C until nonspecific binding was greatly diminished or not visible in comparison with the specific binding, as judged by autoradiography which was performed after each washing using Kodak XAR-5 film. The final wash was performed with 0.4 N NaOH for 30 min at 30 °C in order to strip the filter for use with the next SSO; this way, each filter was used multiple times. In order to ensure that an appropriate amount of amplified DNA was applied on

Primer #	Restriction site	Oligonucleotide sequence (5'-3')	Denature min/°C	Anneal min/°C	Extend min/°C
For DRB					
PZ5A2	Hind III	<u>aaaagc</u> ttACCGGATCGTTCTTGTCCCCCAG ¹⁴ CA ¹⁵	1.5/94	2/52	3/72
PZ2A	Bam HI	ccgga ²⁸² tcctTCGCCGCTGCACTGTGAA ²⁶⁵			
For DRB3					
A52PR	none	¹⁴ CACGTTTCTTGGAGCTGC ³¹	2/94	2/56	1/72
PZ2A		same as PZ2A above			
For DQA1					
GH26	Pst I	gtgctgca ³⁴ GGTGTAAACTTGTACCAG ⁵¹	2/94	2/55	1/72
GH27	Bam HI	cACgga ²⁵⁸ tcGGTAGCAGCGGTAGAGTTG ²⁴⁰			
For DQB1					
DGM4	Bam HI	ttggatcc ¹⁴ AGGATTTCTGTGTA ^t CCAGTTTAAGG ³⁷	1.5/94	2/52	2.5/72
DGM7B [†]	Hind III	ttaagc ²⁴⁹ ttGTAGTTGTGTCTGCACAC ²³²			

Fig. 1. PCR primers used to amplify the second exons of *HLA-DRB*, *-DQA*, and *-DQB* genes. In each set, the first primer is the upstream primer, for which the plus-strand sequence is given, and the second primer is the downstream primer, for which the minus-strand sequence is given. The nucleotide numbering corresponds to cDNA sequence (5' to 3'). Nucleotides shown in *capital letters* constitute the actual primer sequence homologous to the template sequence; nucleotides in *lower-case letters* (not underlined) are necessary for cloning purposes; restriction sites are *underlined*. Primer regions covering intron sequences are shown in *italics*. (The second exons of *DRB* and *DQB* are both comprised of nucleotide residues 14-282, according to cDNA numbering.)

the filter, all the slot-blots were first probed with a framework SSO which was complementary to a conserved region of the *DRB*, *DQA*, or *DQB* second exons (Fig. 2).

Radiolabeling of SSOs for the slot-blot analysis. The oligonucleotides were 5'-labeled with γ - ^{32} P-ATP (≥ 5000 Ci/mmol) in the presence of T4 polynukinase (BRL), followed by column purification using P6 Spin-columns (BioRad, Richmond, Virginia). Specific activities of $1-5 \times 10^9$ cpm/ μ g were obtained for each SSO.

M13 cloning and dideoxy sequencing. In a few cases, the PCR-amplified DNA was cloned into M13mp10 and the single-stranded phage DNA was isolated by standard techniques and sequenced by the dideoxy primer extension method using the Sequenase kit, as suggested by the manufacturer (US Biochemicals, Cleveland, Ohio). The GC-rich regions were resolved by the use of dITP in place of dGTP. Each sequence was confirmed by sequencing several clones. The BIONET computer analysis system (Intelligenetics, Mountain View, California) was used for sequence analyses.

SSO	APR	Specificity	HLA-D genes	Sequence (5'—3')	Td/Tw (°C)
DRB1 Probes:					
A7	1	DR8,12	DRB1*0801,0802,0803, Dw8.3 $\S$,1201,DRw12b $\S$	²⁵ GAGTACTCTACGGGTGAG ⁴²	56/56
A8	1	DR3,5,6	DRB1*0301,0302,DR(new) $\S$, 1101,1102,1103,1104, 1301,1302,1401,1402	²⁵ GAGTACTCTACGTCTGAGT ⁴³	56/56
A21a	2	DR6	DRB1*1301,1302,1401	⁷⁴ GGTTCTCTGGACAGATACTTCCATA ⁹⁷	58.3/62
A22a	2	DR3,52a	DRB1*0301;DRB3*0101	⁷⁴ GGTACCTGGACAGATACTTCCATA ⁹⁷	58.3/61
A28a	4	DR5	DRB1*1101,1104	²⁰⁷ AGACAGGCGGGCCGCG ²²²	58/60
A29	4	DR8	DRB1*0801,0802,0803 Dw8.3 $\S$,DR(new) $\S$	²⁰⁸ GACAGGCGGGCCCTGG ²²³	58/61
A31a	4	DR3	DRB1*0301,0302	²¹⁷ GGCCGGGTGGACAACCTAC ²³⁷	60/64
DRB-FW		all DRB genes		²³⁶ GCAGACACAACCTACGGG ²⁵²	
DRB3 Probes:					
Z15a	3	DR3,4,4,52a,b,c	DRB1*0301,0302,0401; DRB3*0101,0201, 0202,DRw52b2 $\S$,0301	¹⁹⁹ CTCCTGGAGCAGAAGC ²¹⁴	52/54
A15	2	DR52b	DRB3*0201,0202,DRw52b2 $\S$	⁷⁷ TCCTGGAGAGACACTTCC ⁹⁴	56/54
A15xa	2	DR52c	DRB3*0301	⁷⁷ TCCTGGAGAGATACTTCC ⁹⁴	54/54
A22a	2	DR3,52a	DRB1*0301;DRB3*0101	⁷⁴ GGTACCTGGACAGATACTTCCATA ⁹⁷	58.3/58
A32a	4	DR52a	DRB3*0101	²¹⁷ GGCCGGGTGGACAATTAC ²³⁷	58/60
A33a	4	DR52b,c	DRB3*0201,0202,DRw52b2 $\S$, 0301	²¹⁷ GGCCAGGTGGACAATTAC ²³⁷	56/58
DQA1 Probes:					
A205	2	DQ1.1,1.9	DQA1*0101	⁹³ AGATGAGGAGTTCTACGTG ¹¹¹	56/57
A208	2	DQ4,2,3,1,3,22	DQA1*0401,0501,0601	¹⁰⁵ CTACGTGGACCTGGGGAGG ¹²³	64/63
A210	2	DQ1.18	DQA1*0103	¹¹¹ GACCTGGAGAAGAAG ²⁶	46/46
A212	3	DQ4,2,3,1,3,22	DQA1*0401,0501,0601	¹³⁹ TGTTTGCCTGTTCTCAGAC ¹⁵⁷	56/61
A224	5	DQ2,3,1,3,22	DQA1*0501	²²⁰ AACAGTCTGATTAA ²³⁴	38/38
DQA-FW		all DQA1		⁷⁸ CCATGAATTTGATGGAGA ⁹⁵	
DQB1 Probes:					
A101	1	DQ1.1,1.9,1.21	DQB1*0501,0503,0502	²⁴ GTACCAGTTTAAGGGCCTG ⁴²	58/60
A103	1	DQ3.1	DQB1*0301	²⁴ GTACCAGTTTAAGGCCATG ⁴²	56/56
M1	1	DQ1.2,4	DQB1*0602,0401,0402	²⁴ GTTCAGTTTAAGGGCAT ⁴¹	56/60
M19	2	DQ1.1,1.9,1.21	DQB1*0501,0503,0502	⁷⁴ GGGGTGTGACCAGACA ⁶⁹	58/56
M12a	2	DQ1.18,1.19a,b	DQB1*0603,0604	⁷⁵ TCTTGTAAACCAGACACA ⁹¹	48/48
M16	3	DQ1.1,1.19a,b	DQB1*0501,0604,DQ1.7b $\S$	¹⁶¹ GGCGGCCCTGTTGCCGAG ¹⁷⁷	58/61
M2	3	DQ1.2,1.18	DQB1*0602,0603	¹⁵⁶ GCAGGGGCGGCCTGATGC ¹⁷³	64/64
M14	3	DQ1.9,1.12	DQB1*0503,0601	¹⁶¹ GGCGGCCCTGACGCCGAGT ¹⁷⁸	64/66
A119	3	DQ2	DQB1*0201	¹⁵¹ ACGCTGCTGGGGCTGC ¹⁶⁶	56/60
DQB-FW		all DQB1		⁴¹ TGTGCTACTTCACCAA ⁵⁶	

Fig. 2. SSOs used in this study. The letter w that usually accompanies some HLA-DR and -DQ specificities has been eliminated from the figure for clarity. The *HLA* genes are named according to new nomenclature (Bodmer et al. 1990), except for those labeled $\S$, which have not yet been named; these are *DR* "new", *Dw8.3*, *DRw12b*, *DRw52b2*, and *DQ1.7b*.

Nomenclature of HLA-D genes. We will use the new nomenclature for the *HLA-D* genes (e. g., *DRB1*0101* for the *DRB1* gene that encodes the serological specificity DR1; Bodmer et al. 1990). However, the serological specificities DR1, DR2, etc. will be frequently used for convenience.

Antibody determinations. Specific IgG Ab levels were determined by double Ab radioimmunoassays as a part of earlier studies (Ansari et al. 1989b). IgG Ab was determined before, during, and after Rx.

Results

We investigated whether the *Lol p III* responders possessed *HLA-D* sequence(s) generally not found in the nonresponders. This was done by examining the *HLA-D* region DNA sequences from IgG Ab responders and nonresponders, totalling 41 subjects, who had all received grass Rx. We focused on the highly polymorphic regions within the second exons of these genes, since these regions are believed to encode antigen-binding portions of the Ia molecules. Starting with the genomic DNA from the 41 subjects, the second exons of *HLA-DRB*, *-DQA*, and *-DQB* genes were separately amplified by the PCR using specific oligonucleotide primers. After blotting on nylon membranes, these PCR-amplified products were probed with the SSOs shown in Figure 2; the results are shown in Figures 3 and 4.

***DRB1* gene.** Since immune response to *Lol p III* is associated with HLA-DR3, and to some degree with DR5 and DRw6, we used SSOs specific for these three specificities, in addition to DRw8 and a few other alleles (data not shown).

Only SSO # A8 showed a strong correlation with immune responsiveness to *Lol p III*; no such correlation was observed with any other probe. SSO # A8 binds to a DNA sequence encoding the protein sequence E₉YSTS₁₃ found in the first polymorphic region of the DRβI chain of DR3 and also DR11 (split of DR5) and DRw6 (the DRw12 variant of DR5 does not contain this sequence). No other DR (or DQ and DP) polypeptide chains are known to have the EYSTS sequence. Nineteen subjects gave positive signals with this SSO; 18 of them were responders to *Lol p III* before and/or after Rx. The one exceptional subject, # 624, who was positive with SSO # A8 but nonresponder to *Lol p III*, appears to be a relatively poor responder to grass allergens; thus, even after 3 years of Rx with grass pollen extract he continued to be IgG Ab⁻ to the more immunogenic allergen *Lol p I*. Furthermore, in the entire group of 41 subjects, # 624 had the lowest total serum IgE level. DNA sequencing of the *DRB* genes from this subject revealed no unusual features: he had a *DRB1* sequence identical to the known DRw6a type (*DRB1*1301*), and a *DRB3* sequence identical to the known DRw52a type (*DRB3*0101*), both consistent with his serological typing of DRw6.

These data suggest that the sequence EYSTS is important in the presentation of an agretope in *Lol p III*. However, ten subjects lacked the EYSTS sequence (i. e., gave negative signals with probe # A8) but were nevertheless *Lol p III*⁺; other class II molecules of these individuals may be recognizing different agretope(s) of the allergen. The EYSTS sequence was significantly associated with responsiveness to *Lol p III* ($P=0.001$, relative risk=21.6).

***DRB3* gene.** Since DR3, 5, and w6 specificities, which have the sequence EYSTS, belong to the DRw52 family, the DRw52 serological supertypic specificity was also found significantly associated with responsiveness to *Lol p III* ($P=0.008$, relative risk=9.9). This led us to investigate whether the primary association was with the EYSTS sequence found in DRβI chains, or with a sequence in the DRw52βIII chains. We specifically amplified the second exons of the DRw52-associated *DRB3* genes and probed them with DRw52-specific SSOs. For these amplifications, the downstream primer (PZ2A) was the same as that used for the amplification of *DRB1* genes, but the upstream primer (A52PR) was complementary to the first allelic polymorphic region of *DRB3* genes. In this region, all the *DRB3* genes have identical sequences but differ from other *DRB* genes. Therefore, we were able to amplify specifically the *DRB3* genes in the absence of any detectable amplification of other *DRB* genes. The specificity of this amplification was checked by the use of two SSOs: DRB-FW that hybridizes with all *DRB* genes, and Z15a that hybridizes primarily with *DRB3* genes (and also with *DRB1*0301* and *DRB1*0401*). Both the SSOs gave positive signals in all cases having the DRw52a, b, or c specificity, but in no case that was devoid of the DRw52 specificity. Having seen that in each case *DRB3* genes and no other *DRB* genes were amplified, we probed the amplified DNA with SSOs for the genes encoding the specificities DRw52a (*DRB3*0101*), DRw52b (*DRB3*0201*, *0202*, and *0203*), and DRw52c (*DRB3*0301*). None of the SSOs gave a strong relationship with responsiveness to *Lol p III*. Five different sequences of DRβIII chains are known: one each for DRw52a and DRw52c (*DRB3*1001* and *0301*, respectively), and three for DRw52b (*DRB3*0201*, *0202*, and *0203*); the three latter sequences are identical in their hypervariable regions. Except for one subject (ID # 1112) who was DRw52b/52c, all our EYSTS⁺ subjects (or DRw52⁺ subjects) were found by SSO analysis to be either 52a (ten subjects), 52b (five subjects), or 52a/52b (four subjects). Thus, 52a or 52b subjects were both *Lol p III* responders. But not a single hypervariable region is identical in 52a and 52b β chains. These observations strongly indicate that no specific DRw52 sequence may be the *Lol p III* responder sequence, and that the DRw52 association with responsiveness may be secondary to the

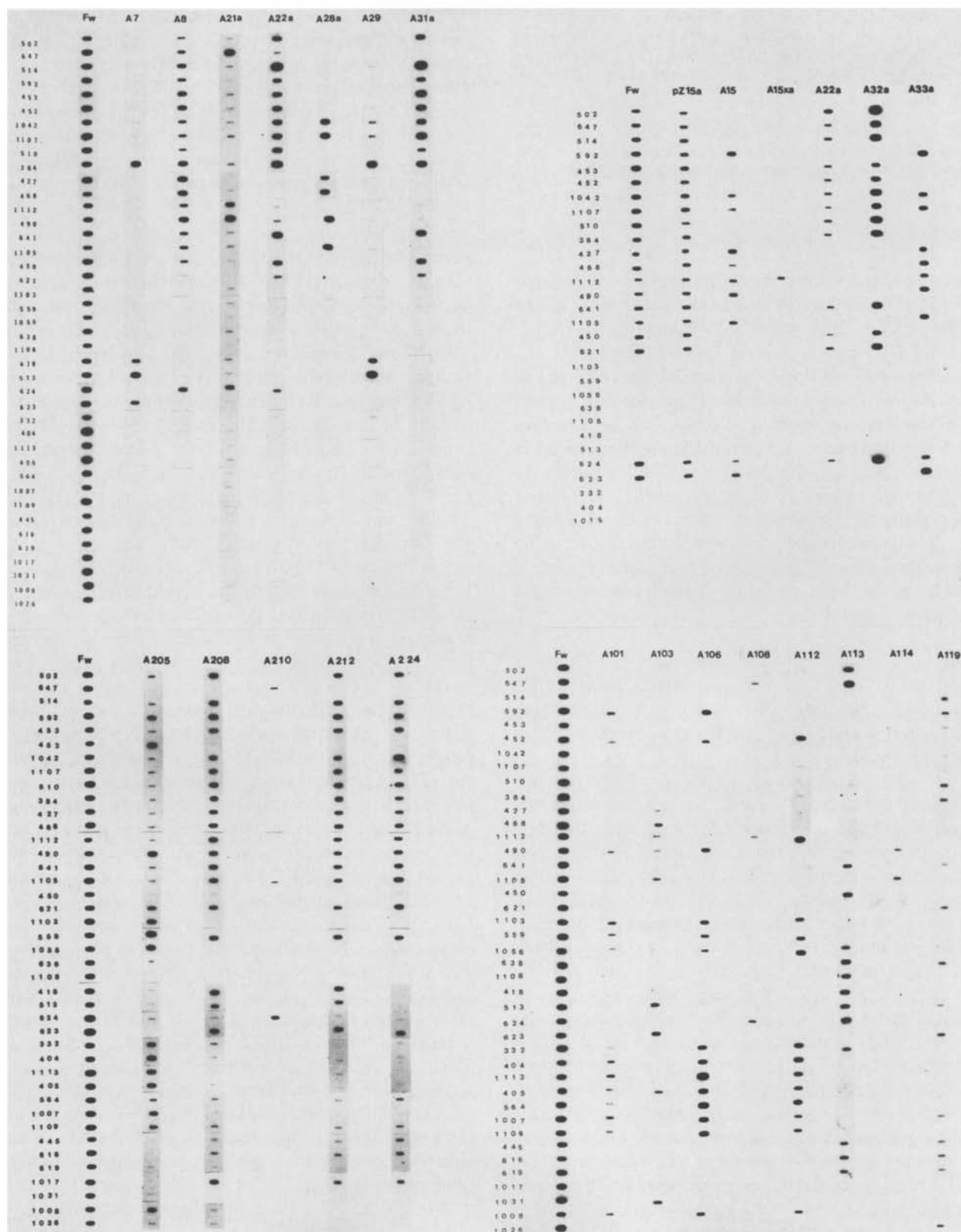


Fig. 3. Slot-blot results with *DRB1*, *DRB3*, *DQA1*, and *DQB1*.

		Gene		DRB1 APR1		DRB1 APR2		DRB1 APR4			DRB3 APR2			DRB3 APR4			DQA1 APR2			DQA1 APR3		DQA1 APR5		DQB1 APR1		DQB1 APR2		DQB1 APR3					
		SSO #		A7	A8	A21a	A22a	A28a	A29	A31a	Z15a	A15	A15xa	A22a	A32a	A33a	A205	A208	A210	A212	A224	A101	A103	M1	M19	M12a	M16	M2	M14	A119			
Old ID	New ID	DR	DR	R3 resp	8	3	6	3	5.6	8	3	52a	52b	52c	3	52a	52b	101	601	103	601	601	501	501	501	301	401	601	603	501	602	603	201
					6		52a					52b			52a	52b	52c							502		602	602	604	604	603	601		
MB	502	2	3	+	-	+	-	+	-	-	+	+	-	-	+	+	-	±	+	-	+	+	-	-	-	-	-	-	-	+	-	+	
JS _{ti}	647	2	6	+	-	+	+	-	-	-	-	+	-	-	+	-	-	±	+	-	+	+	-	-	-	-	-	-	+	-	-	-	
PL	514	3	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	
CLo	592	3	2	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
LBa	453	3	4	+	-	+	-	+	-	-	+	-	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
MBa	452	3	4	+	-	+	-	+	-	-	+	-	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
BSh	1042	3	5	+	-	+	-	+	+	-	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	
RMol	1107	3	5	+	-	+	-	+	-	-	+	+	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
WSc	510	3	7	+	-	+	-	+	-	-	+	+	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
RV	384	3	8	+	+	-	-	-	-	+	+	-	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
WL	427	5	4	+	-	+	-	-	+	-	-	+	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
SG	468	5	4	+	-	+	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
CS _t	1112	5	6	+	-	+	+	-	-	-	+	+	-	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
RDa	490	5	6	+	-	+	+	-	+	-	-	+	+	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
RH _i	641	2	3	(+)	-	+	-	+	-	-	+	+	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
SF	1105	2	5	(+)	-	+	-	+	-	-	+	+	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
JMc	450	3	4	(+)	-	+	-	+	-	-	+	+	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
LLa	621	3	7	(+)	-	+	-	+	-	-	+	+	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
CB	1103	1	4	+	-	+	-	-	-	-	-	-	-	-	-	-	-	±	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
LL	559	1	4	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
EK _i	1056	2	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	
WR	638	2	7	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	
RMor	1108	2	7	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	
CS	418	2	8	+	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	
EE	513	8	7	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	
MBr	624	6	-	-	+	+	-	-	-	-	-	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
BDa	623	7	12	-	+	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
JG	332	1	2	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
CLi	404	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
JWa	1113	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
DHa	405	1	4	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
VH	564	1	4	+	-	+	-	-	-	-	-	-	-	-	-	-	-	±	±	-	±	±	±	±	±	±	±	±	±	±	±	±	
WFr	1007	1	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	
RCh	1109	1	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	±	-	±	±	±	±	±	±	±	±	±	±	±	±	
CSz	445	2	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	±	±	±	±	±	±	±	±	±	±	±	±	
JK	616	2	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	±	±	±	±	±	±	±	±	±	±	±	±	
GD	619	2	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	±	±	±	±	±	±	±	±	±	±	±	±	
QM	1017	2	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	±	±	±	±	±	±	±	±	±	±	±	±	
GBa	1031	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
LBa	1008	4	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
JL	1026	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Fig. 4. Summary of the slot-blot results. R3 resp is the IgG Ab response to *Lol p* III. + indicates responder after Rx.

association with the EYSTS sequence. This conclusion is further supported by subject #623, who is DRw52⁺ at the *DRB3* locus but lacks the EYSTS sequence at the *DRB1* locus and is nonresponder to *Lol p* III.

***DQA1* gene.** Because of the linkage disequilibrium between *DR* and *DQ*, we also considered the possibility that the primary association of immune responsiveness to *Lol p* III might rather be with a sequence in *DQA1* or *DQB1*, the only expressed genes in the *DQ* region. The *DQA1* genes generally found in DR3, 5, and w6 subjects (i. e., those having the EYSTS sequence in the *DRβ1* polypeptide chain) are as follows: both DR3 and DR5 subjects have the *DQA1* gene *DQA1*0501*, while DRw6 is normally associated with any of the three *DQA1* genes *DQA1*0101*, *0102*, and *0103*. Thus *DQA1*0501*, being present in two (DR3 and DR5) of the three DR types having the EYSTS sequences, would be the most likely gene in the *DQA* locus to be associated with immune responsiveness to *Lol p* III. Slot-blot analysis of the amplified *DQA1* genes using three SSOs for *DQA1*0501* showed no relationship with responsiveness to *Lol p* III. Neither did SSOs for *DQA1*0101* and *0103*.

***DQB1* gene.** The *DQB1* genes generally found in Caucasoid DR3, 5, and w6 subjects (i. e., those having

the EYSTS sequence in the *DRβ1* polypeptide chain) are as follows: DR3 is associated with *DQB1*0201*, DR5 with *DQB1*0301*, and DRw6 with any of the four *DQB1* alleles *DQB1*0301*, *DQB1*0503*, *DQB1*0603*, and *DQB1*0504*. Thus, *DQB1*0301* is the only *DQB1* gene that is occasionally shared among the DR5 and DRw6 subjects. However SSO #A103, specific for this gene, did not show any relationship with immune response to *Lol p* III. Neither did other SSOs for *DQB1* genes.

Relationship between EYSTS and immune response to *Lol p* III. This relationship is depicted in Figure 5, each curve representing the *Lol p* III IgG Ab level of a single patient over the course of Rx with mixed grass pollen antigens. All the 19 EYSTS⁺ subjects are shown in panels A and B. Panel A shows those EYSTS⁺ subjects who were *Lol p* III IgG Ab⁺ to natural grass exposure (before Rx). As expected, the specific IgG Ab level in most patients rose during the course of Rx. Panel B shows those EYSTS⁺ subjects who were *Lol p* III IgG Ab⁻ before Rx. Four of these five subjects became *Lol p* III IgG Ab⁺, leaving only one EYSTS⁺ subject who did not respond to *Lol p* III. The results for all the EYSTS⁻ subjects are shown in panels C and D. Panel C shows those EYSTS⁻ subjects who were *Lol p* III IgG Ab⁺ before Rx. Panel D shows EYSTS⁻ subjects who were nonresponders before Rx.

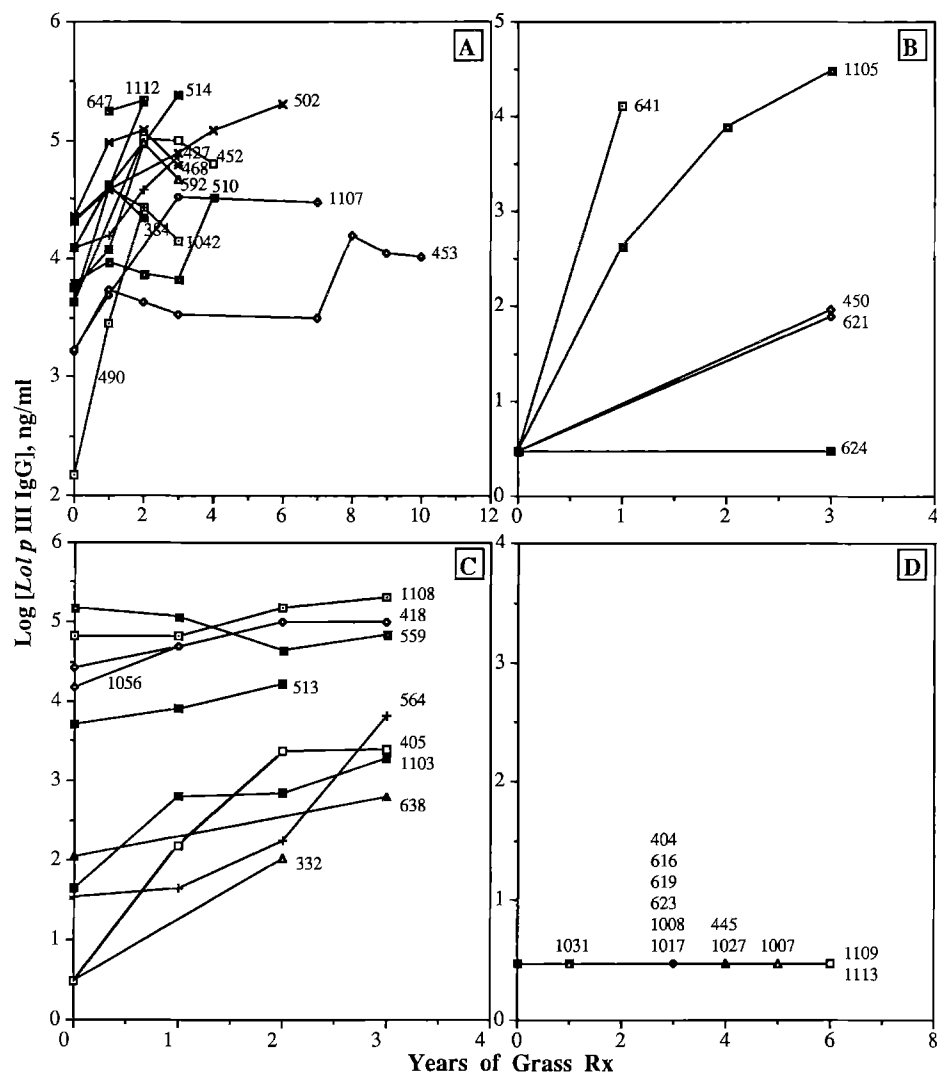


Fig. 5. *Lol p* III Ab response of the 41 patients during the course of Rx with grass pollen extract. Panels A and B include all the EYSTS⁺ patients who, before Rx, were *Lol p* III Ab⁺ (A) or *Lol p* III Ab⁻ (B). Panels C and D include all the EYSTS⁻ patients who, before Rx, were *Lol p* III Ab⁺ (C) or *Lol p* III Ab⁻ (D).

Discussion

The role of major histocompatibility complex (MHC) genes is controlling the host's immune responsiveness, and the resulting statistical associations between specific HLA-D types and immune responses to various antigens (Marsh et al. 1982a, b; Goodfriend et al. 1985; Freidhoff et al. 1988; Ansari et al. 1989a, b) or between HLA-D types and various autoimmune disorders (Tiwari and Terasaki 1985) are well recognized. The molecular basis of these associations is, however, poorly understood. In this paper, we have demonstrated a striking association between a specific *HLA-D* sequence and immune responsiveness to a well-defined antigen, *Lol p* III. This "responder sequence", E₉YSTS₁₃, is present in the first polymorphic region of DRβI peptide chains found in DR3, DR11 (split of DR5), and DRw6 individuals. All but one

(18/19) EYSTS⁺ subjects were *Lol p* III responders as measured by specific IgG Ab; 10 of 22 EYSTS⁻ subjects were also *Lol p* III responders ($P=0.001$, relative risk=21.6). We also found that none of the *DRB3* sequences of the supertypic specificity DRw52, found in linkage disequilibrium with DR3, DR5, and DRw6, was associated with immune responsiveness to *Lol p* III. Also, *DQA1* or *DQB1* sequences, found in linkage disequilibrium with DR3, DR5, or DRw6, were not associated with immune responsiveness. The *Lol p* III molecule has a relative mass of 11 000 (Ansari et al. 1989c) and most likely contains more than one epitope; this would be consistent with our hypothesis that one of the epitopes is recognized by the *HLA-D* sequence EYSTS, while other *HLA-D* sequences may be important in the recognition of other epitopes of *Lol p* III. The strong association with EYSTS was not entirely predictable from the earlier

studies (Ansari et al. 1989a, b) or from sequence data because of linkage disequilibrium between the alleles of *DRB1*, *DRB3*, *DQA1*, and *DQB1* loci.

Previously, specific *HLA-D* residue positions or sequences have been implicated in susceptibility to certain autoimmune disorders, including insulin-dependent diabetes mellitus (Todd et al. 1987, 1988; Horn et al. 1988; Ronningen et al. 1989a, b; Sheehy et al. 1989; Yamagata et al. 1989; Awata et al. 1990; Khalil et al. 1990), pemphigus vulgaris (Scharf et al. 1988; Todd et al. 1988), and rheumatoid arthritis (Todd et al. 1988; Winchester 1990). However, the present report represents the first demonstration of a strong association between a specific *HLA-D* sequence and immune responsiveness to a well-defined antigen. Identification of MHC sequences critical for specific antigen presentations is important for designing peptide-based immunotherapeutic approaches.

These results do not however demonstrate a cause-effect relationship, which must await a detailed study using antigen-specific T-cell clones and antigen-presenting cells, including transfected cell lines, of various *HLA-D* phenotypes. Nevertheless, the sequence EYSTS might provide an important antigen-binding region, as it forms part of the "floor" of the antigen-binding groove in the model proposed for MHC class II molecules (Brown et al. 1988). Interestingly, Sjögren's syndrome is also associated with *HLA-DR3*, *-DR5*, and *-DRw6* (E. Alexander, personal communication), and it is conceivable that the EYSTS sequence may be important in this autoimmune disorder. Residue position 9 in this E₉YSTS₁₃ sequence may be critical, as substitution of this residue from Glu to Lys in the *DR3β* chain selectively altered antigen presentation of tetanus toxoid (Mellins et al. 1988).

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