

DR7 and DQ2 Are Positively Associated with Immunoglobulin-E Response to the Main Antigen of Olive Pollen (*Ole* e I) in Allergic Patients

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ABSTRACT: We have studied the relationship between HLA class II haplotypes and alleles, and the IgE antibody response to a highly purified allergen, *Ole* e I, in allergic patients. *Ole* e I, is the major antigen from the pollen of olive tree that grows mainly in the Mediterranean. Genomic DNA typing was performed in 40 unrelated patients with seasonal allergic pollenosis who had specific IgE antibodies against *Ole* e I, detected by double-antibody radioimmunoassay. HLA-DRB and -DQB loci were analyzed by PCR-SSO and RFLP. Phenotypic fre-

quencies were compared with those of 179 healthy unrelated individuals. Significant increases in the phenotypic frequencies of DR7 (pf = 67.5% vs 31.3% in the control population, $p_c = 0.0023$) and DQ2 (pf = 90.0% vs 48.0%, $p_c = 0.0003$) were found, indicating an association between DRB1*0701/2, DQB1*0201 alleles and the IgE antibody response to *Ole* e I. This is the first time that the HLA-DQ gene has been associated with a positive allergic response. *Human Immunology* 38, 293-299 (1993)

ABBREVIATIONS

cpm counts per minute
gf gene frequency
Ig immunoglobulin
mAb monoclonal antibody
MHC major histocompatibility complex
PBMC peripheral blood mononuclear cells

p_c corrected probability
PCR polymerase chain reaction
pf phenotypic frequency
RFLP restriction fragment-length polymorphism
RR relative risk
SSO sequence-specific oligonucleotide

INTRODUCTION

Atopic allergy is an excellent model for the analysis of the genetic basis of the human immune response [1].

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One of the principal advantages is the fact that some causative antigens are well characterized and, in some cases, have been produced by recombinant DNA technology.

The polymorphic class II products of the major histocompatibility complex (MHC) have an important function in the presentation of antigen fragments to the T-cell receptor, triggering the immune response that leads to immunoglobulin (Ig)-E antibody production and allergy in genetically susceptible humans.

In the last 20 years, different associations have been

described between HLA haplotypes and autoimmune disorders [2]. These associations have been defined mostly by serologic techniques, but in the last few years, DNA methodology has enhanced the possibilities of defining disease susceptibility to an allelic level [3, 4]. Several studies have shown positive associations between particular class II phenotypes and the antibody response to certain highly purified allergens [5–7]; however, some reports show a protective role for determined haplotypes, as in the case of DQ and the response to *Cryptomeria japonica* pollen [8, 9].

Allergy to olive pollen is one of the main causes of allergic diseases in Mediterranean countries [10]. In 1988, we reported [11, 12] the isolation and physicochemical characterization of two major allergens derived from this pollen: *Ole* e I (19 kD) and *Ole* e II (8 kD). The N-terminal amino acid sequence of *Ole* e I has also been reported [13].

In this report, DNA typing by polymerase chain reaction (PCR)—sequence-specific oligonucleotide (SSO) and restriction fragment-length polymorphism (RFLP) shows strong associations between DR7 and DQ2 antigens and the immune response to *Ole* e I in allergic patients, this being the first time that a positive response has been described in relation with DQ alleles.

MATERIALS AND METHODS

Patients and Controls

A population of 97 Caucasoid unrelated allergic patients, with positive skin tests and serum IgE antibodies to *Olea europea* crude extract and to other common allergens (detected by radioallergosorbent test, RAST), was selected for antibody studies.

From the 60 patients with serum IgE antibodies against *Ole* e I, we used a group of 40 that came for the second visit, in order to perform the DNA typing.

A total of 179 unrelated healthy subjects were used as a control population.

Antibody Measurement and Serum IgE Levels

Specific IgG and IgE antibodies to highly purified allergen *Ole* e I were measured in duplicates by double-antibody radioimmunoassay (DARIA) [14]. *Ole* e I was radiolabeled with ^{125}I by using the chloramine-T method. Goat-anti-human IgE and anti-human IgG were kindly provided by D. G. Marsh (Johns Hopkins University, Baltimore, MD, USA) and IgE myeloma PS by K. Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA).

In regard to sera IgG antibodies, a serum was considered to be positive when the binding was above 1.3 times the value obtained with sera from healthy people

with no known allergies (1400 ± 300 counts per minute [cpm]).

Specific IgE antibodies were measured in the sera of patients with IgG antibodies to *Ole* e I. Specific IgE serum values were expressed in arbitrary units by reference to a standard curve based on the serum with the maximal IgG binding.

Total serum IgE was measured by Pharmacia IgE enzyme immunoassay (Uppsala, Sweden).

Antigen Purification

Ole e I was obtained after gel filtration and HPLC purification by reverse-phase chromatography of crude olive pollen extract (Allergon, Angelholm, Sweden), following a previously described method [13].

Cell Cultures

Peripheral blood was obtained from two *Olea europea*-allergic individuals (DB and GMZ), previously typed. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway). After three washes in RPMI 1640, the PBMCs were resuspended in culture medium RPMI 1640 supplemented with 10% fetal calf serum, 4 mM L-glutamine, 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 1 mM sodium pyruvate (Flow Laboratories, Irvine, Scotland). PBMCs (3×10^5 cells/200- μl well) were cultured in triplicate in 96-well microtiter plates, in the absence or presence of concanavalin A (5 $\mu\text{g}/\text{ml}$) or *Ole* e I antigen (5 $\mu\text{g}/\text{ml}$) at 37°C and 5% CO_2 in humidified air. After 5 days of culture [15], [^3H]thymidine (New England Nuclear, Boston, MA, USA) was added, and its incorporation was measured after 16–18 hours in a β -scintillation counter (Beckman LS 2800; Palo Alto, CA, USA).

In the inhibition assays, 5 $\mu\text{g}/\text{ml}$ of anti-human MHC class II monoclonal antibodies (mAbs) specific for the nonpolymorphic region of DR and DQ was dialyzed against culture medium and added at the initiation of the culture [16]. The mAb used for DR inhibition was murine IgG3 antibody (MAB1762) purified from ascites and for DQ murine mAb IgG1 (MAB1764), also purified from ascitic fluid. Both were purchased from Chemicon International (Temecula, CA, USA).

DNA Extraction

Genomic DNA was extracted from 20 ml of peripheral blood following the salting-out method [17], with minor modifications.

PCR-SSO

DNA amplification. Genomic DNA was used as template for amplification of the DRB and DQB1 second-exon gene segments. DRB alleles were amplified with

specific primers of HLA-DRB1, -B3, -B4, and -B5 [18]: DRB-AMP.A (5'-CCCCACAGCACGTTTCTTG-3') and DRB-AMP.B (5'-CCGCTGCACTGTGAAGCTCT-3'). PCR was carried out as described by Kimura and Sasazuki [19]: 30 cycles of denaturation for 1 minute at 95°C, annealing for 1 minute at 55°C, and extension for 2 minutes at 72°C.

The primers for HLA-DQB amplification were DQB-AMP.A (5'-CATGTGCTACTTCACCAACGG-3') and DQB-AMP.B (5'-CTGGTAGTTGTGTCTGCACAC-3'). The same amplification conditions as for HLA-DRB were used. Primers were provided by Dr. T. Sasazuki in the context of the 11th International Histocompatibility Workshop.

All PCRs were done using 1.5 U Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) in a reaction volume of 50 μ l with the following reagents: 0.5 μ g of genomic DNA, 25 pmol of each primer, 20 nmol of all four dNTPs in PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5–2.5 mM MgCl₂, and 0.1% [wt/vol] gelatin).

The amplification reactions were carried out in a PREM thermal cycler (Lep Scientific, Milton Keynes, England).

Dot blot of PCR products. A total of 3 μ l of amplified DNA sample was denatured with 50 μ l of 0.4 N NaOH and 25 mM EDTA for 10 minutes at room temperature, and 50 μ l of the mixture was spotted onto a nylon membrane (Gene Screen; NEN Products, Boston, MA, USA) prewetted in distilled water using an Easy-Titer ELIFA system (Pierce, Rockford, IL, USA) dot-blot apparatus. The DNA was immobilized by drying the filter at 80°C for 1 hour. Duplicate membranes were prepared.

Oligonucleotide probes. SSOs used for HLA typing [19] were provided by the 11th International Histocompatibility Workshop for the HLA and Allergy Project, chaired by Dr. D. G. Marsh. A total of 24 SSO probes designed for generic DRB typing and SSOs DQB 5705 and 7005 (both specific for DQB1*0201) were used.

To ensure that an appropriate amount of amplified DNA was applied to the filter, all of the membranes were first probed with framework SSOs that were complementary with a conserved region of DRB (SSO-1 and SSO-2) and DQB (SSO-1) second exons.

Hybridization and detection. The filters were wetted in 4 $\times$ SSPE solution (0.6 M NaCl, 0.04 M NaH₂PO₄, and 4 mM EDTA, pH 7.4) and prehybridized at 54°C for at least 30 minutes in 10 ml of TMAC solution (50 mM Tris-HCl pH 8.0, 3 M tetramethylammonium chloride, 2 mM EDTA, and 0.1% SDS) containing 5 $\times$ Denhardt's solution and 100 μ g/ml heat-denatured her-

ring-sperm DNA. Then, 10 pmol of SSO (5'-labeled with [γ -³²P]ATP, >7000 Ci/mmol, in the presence of T4 polynucleotide kinase) was added and hybridization was continued for 1–3 hours at 54°C in a water bath with constant shaking. Filters were washed twice in 2 $\times$ SSPE containing 0.1% SDS at room temperature for 10 minutes, once in TMAC solution for 10 minutes at room temperature and twice in the same solution at 57°C for 10 minutes. After the last washing, x-ray film (Kodak, XAR-5) was exposed with an intensifying screen to the membrane at –70°C for 1–16 hours.

RFLP. Taq I (and MspI, when necessary)-digested genomic DNAs were electrophoretically separated in agarose gel and vacuum blotted onto nylon filters, which were hybridized with ³²P-labeled cDNA for HLA-DRB [20], -DQA [21], and -DQB [22] loci according to standard techniques [23].

Haplotype assignment. DRB typing by PCR-SSO [19] was used to classify individuals in generic DR groups. DR-DQ haplotypes were assigned by RFLP according to the methods described by Bidwell et al. [23] and Martell et al. [24], and the DQB1*0201 allele was definitively assigned following hybridization with the SSO probes previously described.

Statistical analysis. Statistical significance of differences in antigen frequencies between patients and controls was measured by the χ^2 test (with the Yates correction), and the probability was corrected (p_c) for the number of antigens studied. Relative risk (RR) and either etiologic or preventive fractions were calculated, when appropriate, according to the method described by Svejgaard et al. [25].

RESULTS

Antibody results. Specific IgG antibodies against *Ole e* I, the major antigen of the olive tree pollen, were detected in 72 (75%) of 97 allergic patients sensitized to *Olea europea* crude extract. Patients positive for specific IgG were analyzed for the presence of specific IgE antibodies, which were found in 83% ($n = 60$). HLA class II typing was done in 40 patients who had specific IgE antibodies and who came for the second visit.

DNA typing. Phenotypic frequencies of DR and DQ antigens in the allergic and control groups are listed in Table 1. A statistically significant increase ($p_c = 0.0023$) in the phenotypic frequency of DR7 (67.5%) in the patient population was found when it was compared with the control group (31.3%). A very highly significant increase ($p_c = 0.0003$) in the phenotypic frequency

TABLE 1 PCR-SSO and RFLP-deduced phenotypic frequencies of DR and DQ antigens in allergic patients with specific IgE against *Ole* e I and in controls

	Patients ^a	Controls ^b	<i>p</i> ^c	RR	EF/PF ^e
DR1	15.0	21.2	NS		
DR2 (15)	10.0	21.2	NS		
DR2 (16)	0.0	3.4	NS		
DR3	35.0	25.1	NS		
DR4	10.0	30.2	NS ^d	0.26	0.22
DR5 (11)	17.5	18.4	NS		
DR5 (12)	2.5	1.7	NS		
DR6 (13)	15.0	22.3	NS		
DR6 (14)	2.5	3.4	NS		
DR7	67.5	31.3	0.0023	4.56	0.53
DR8	0.0	6.1	NS		
DR9	2.5	2.2	NS		
DR10	2.5	2.2	NS		
DQ1 (5)	17.5	31.3	NS		
DQ1 (6)	25.0	38.5	NS		
DQ2	90.0	48.0	0.0003	9.73	0.81
DQ3 (7)	25.0	30.7	NS		
DQ3 (8)	5.0	22.3	NS ^d	0.18	0.19
DQ3 (9)	7.5	5.6	NS		
DQ4	0.0	5.6	NS		

^a Patients, 40 allergic patients with serum IgE antibodies anti *Ole* e I.

^b Control population, 179 unrelated healthy subjects.

^c The *p* value was corrected for 13 DR and seven DQ broad antigens, respectively.

^d Uncorrected *p* < 0.05 in these cases.

^e Either etiologic or preventive fractions (EF/PF) were calculated for antigens with RRs >1 or <1, respectively.

of DQ2 (an antigen associated with DR7) was also observed among the specific IgE responders (90% vs 48%). High RRs of 4.56 and 9.73 are respectively conferred by DR7 (DRB1*0701/2 alleles) and DQ2 (DQB1*0201 allele), their etiologic fractions being 0.53 and 0.81.

TABLE 2 Mean serum values of total IgE, specific IgG, and IgE anti-*Ole* e I in patients classified according to their DR, DQ phenotypes

Serum values	DR7-DQ2 (<i>n</i> = 26)	DR3-DQ2 Non-DR7 (<i>n</i> = 10)	Non-DQ2 (<i>n</i> = 4)
Total IgE (IU/ml)	735.4 ± 91.4	552.1 ± 129.3	293.5 ± 109.5
Specific IgG ^a	1446.8 ± 460.8 ^c	1761 ± 776.2	4574.5 ± 1751 ^c
Specific IgE ^b	10.08 ± 1.7	6.18 ± 0.7	5.28 ± 0.6

^a Percent binding of IgG: (test cpm/background normal sera cpm) × 100.

^b IgE binding expressed in terms of arbitrary units per milliliter by reference to a standard curve performed with a serum with the maximal IgG binding.

^c *p* < 0.05.

Also noteworthy, although nonsignificant, is the increase in the frequency of DR3, an antigen also associated with DQ2.

DR4 and DQ8 are remarkably underrepresented among the allergic patients, but the significance of this difference (*p* < 0.05) disappears when *p* is corrected for the number of antigens studied. No other significant differences in antigen frequencies were found.

For comparative purposes, specific IgE responders were classified into three groups according to the presence or absence of the HLA risk factors analyzed: DR7-DQ2 positive, DR3-DQ2 (non-DR7), and non-DQ2. Their respective mean values of total serum IgE, specific IgG, and specific IgE are listed in Table 2. A graduation in the antibody levels was observed in which DR7-DQ2 and non-DQ2 groups showed extreme values: total and specific IgE were highest among DR7-DQ2 positives, whereas specific IgG was highest among DQ2 negatives. DR3-DQ2 (non-DR7) individuals displayed intermediate levels.

Cellular experiments. Functional experiments were performed using PBMCs from two donors sensitized to olive pollen: DB (DR7-DQ2 DR7-DQ2) and GMZ (DR3-DQ2 DR1-DQ5).

In a dose-response curve with different concentrations of purified *Ole* e I antigen, the maximum proliferative response was observed with 5 µg/ml in both patients. Also, cells from a healthy control and one allergic unrelated patient did not respond to *Ole* e I under the same conditions (data not shown).

A total of 5 µg/ml of antigen was used in the inhibition studies using as inhibitors murine mAbs against human MHC class II nonpolymorphic determinants (α-DR and α-DQ).

Figure 1 shows the inhibition of the proliferative response to *Ole* e I produced by α-DR (75% in DB and 54% in the case of GMZ) and by α-DQ (99% in DB

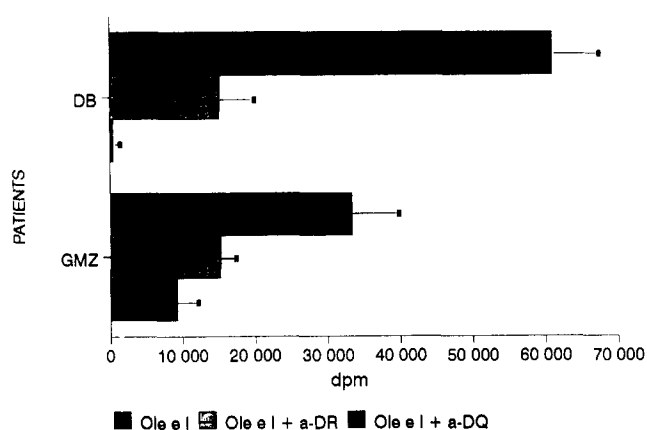


FIGURE 1 Proliferative response of PBMCs from two allergic patients DB (DR7-DQ2, DR7-DQ2) and GMZ (DR3-DQ2, DR1-DQ5) to 5 µg/ml of purified *Ole e I* antigen and inhibition of the response with two monoclonal antibodies against MHC class II nonpolymorphic determinants: anti-DR and anti-DQ (5 µg/ml). Results are expressed as mean disintegration per minute (dpm) $\pm$ SD of triplicate cultures. (Cells without antigen 356 ± 76 dpm.)

and 71% in GMZ). The mitogenic response to concanavalin A (5 µg/ml) was not inhibited with the same concentration of mAbs.

When an unrelated mAb was used as inhibitor (monomeric anti-class-I, w6/32), no inhibition was found (data not shown).

DISCUSSION

We have described a significant association between the IgE antibody response to a highly purified allergen from the olive tree pollen (*Ole e I*) and the HLA class II antigens DR7 and DQ2 in patients with seasonal allergic pollenosis.

Several associations between HLA class II haplotypes and allergic responses have been published: DR2/Dw2 in the case of *Amb a V*, *Amb t V*, and *Amb p V* [14, 26], and DR3/Dw3 with *Lol p I*, *Lol p II*, and *Lol p III* [27, 28]. An association with DR7 has been described in the case of the allergic response to *Jun a I*, the major antigen derived from the pollen of another tree, the mountain cedar [29].

To our knowledge, this is the first report of an association between HLA-DQ genes and the positive response to an allergen. This strong association between DQ2 and IgE antibody-response anti-*Ole e I* induces a high RR (9.73) and an etiologic fraction of 0.81 (Table 1).

Regarding which antigen, DR7 or DQ2 (or both), is responsible for the genetic risk of the disease, no conclusive answer can be ascertained because of the strong

linkage disequilibrium between both antigens, but some aspects point to DQ2 as the main HLA-susceptible factor: (a) The significance of the increased frequency, RR, and etiologic fraction is greater for DQ2 than for DR7, although these values are influenced by the overall higher frequency of DQ2. (b) There is a nonsignificant increase (35% vs 25.1%) in the phenotypic frequency of DR3. If individuals with the DR7-DQ2 haplotype are excluded, the increase in the DR3-DQ2 frequency is even more evident: 10 (71.4%) of the 14 remaining patients carry this haplotype, whereas it is present in only 37 (28.5%) of 130 control individuals.

These data suggest that DQ2 is the antigen primarily overrepresented, whereas the frequencies of DR7 and DR3 are secondarily increased due to their linkage to DQ2 (although DQA alleles have not been determined by PCR-SSO, we know from RFLP data that the phenotypic frequencies of these antigens are within the expected values). Nevertheless, it cannot be excluded that both DR7 and DQ2 are operating jointly as susceptibility factors for the allergy to the pollen of *Olea europaea* (Table 2). Whichever antigen is primarily implicated, this susceptibility trait does not seem to be recessive, since the number of homozygous individuals for DR7 is four (10%) and that for DQ2 is eight (20%), and these values are lower than expected ($gf^2 = 15\%$ and 30.2% , respectively), considering the genotypic frequencies of both antigens ($gf = 38.75\%$ and 55.0%).

The cellular experiments implicate both antigens, since the inhibition produced by the anti-DQ is practically 100% in the case of the DR7-DQ2-homozygous patient (DB), as is shown in Fig. 1. Further experiments should be performed using specific anti-DR7 and anti-DQ2 antisera in order to clarify the main antigen implicated in this response.

A remarkable decrease in the phenotypic frequencies of DR4 haplotypes in allergic patients was found. Although its significance is borderline (uncorrected $p < 0.05$), this low frequency cannot be explained merely as a consequence of the high frequencies of DQ2-associated haplotypes. In contrast, allergic patients sensitized to olive, but nonresponsive to *Ole e I* (with no detectable specific IgG nor IgE), show a DR4 frequency (data not shown) close to that of the control group. These data suggest a possible protective role for the HLA-DR4 antigen or haplotypes.

An interesting feature is that this kind of association between nonresponders and HLA-DR4 has been described for the mountain cedar allergy in the United States [30] and for Japanese cedar (*Cry J I*), which cross-reacts with mountain cedar [31]. In 1991, a negative association was described between DQw3 and the IgG response to this allergen [8]. As described above, there is also an association between the IgE response to

Jun a I and HLA-DR7 in allergic patients [29]. It will be very interesting to compare the amino acid sequences of these allergens when they become available.

Cross-allergenic reactivity between the different Oleaceae family members—*Ligustrum*, *Fraxinus*, and *Syringa*—has been described [10]. All of the sera from our patients with specific IgE to *Ole e I* cross-reacted with the other members of the Oleaceae family, obtaining the strongest reactivity with *Fraxinus* (data not shown). It would be very interesting to study HLA class II haplotypes from allergic patients in areas (Central Europe) where the pollens of the other members of the Oleaceae family are prevalent, in order to determine whether DR7, DQ2, or both are implicated in the antigen presentation.

The association between HLA and allergy presented in this study underlines the need to study HLA phenotypes in patients allergic to these Oleaceae species. This is the first time that an association between HLA and allergy to olive pollen has been described.

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