

Immunogenetics of atopic asthma: association of DRB1*1101 DQA1*0501 DQB1*0301 haplotype with *Dermatophagoides* spp.-sensitive asthma in a sample of the Venezuelan population

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Summary

Background Genes linked to the major histocompatibility complex (MHC), have been implicated in atopic asthma. Asthma is highly prevalent in the Venezuelan population (estimated at 20%) and genetic markers are needed to identify populations at risk and plan intervention strategies.

Objective To study the influence of the MHC class I and class II genes in the susceptibility to atopic asthma.

Methods MHC-class I HLA-A, -C, -B and MHC-class II HLA-DR, -DQ, -DP gene haplotype frequencies were determined in 135 Venezuelan mestizos, 71 belong to 20 atopic asthmatic families and 64 unrelated controls. The index cases were 20 atopic asthmatics with positive skin-prick tests and specific serum immunoglobulin E (IgE) for *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f). To ascertain the genes associated with susceptibility to atopy and/or asthma, two control groups were studied, 41 non-atopic subjects with skin-prick negative test, and undetectable levels of specific IgE and 23 non-atopic subjects with detectable specific IgE to Der p and Der f. A linkage analysis was performed in those families with two or more atopic siblings (with or without asthma).

Results MHC-class I genes analysis showed that HLA-Cw7 was absent in the asthmatic patients studied, whereas the frequency of this allele was 14.3% in non-atopic controls ($P = 0.017$, $PC = 0.19$) and 20.8% in the atopic controls ($P = 0.0066$, $PC = 0.07$). MHC-class II gene analysis showed a significant increase of the HLA-DRB1*11 in the asthmatic patients compared with non-atopic controls (allele frequencies of 25.6 vs 4.4% $P = 0.0017$, $PC = 0.02$). There were no significant differences among asthmatic and atopic controls in the frequency of HLA-DRB1*11 (25.6 vs 17.4%). In contrast, the HLA-DRB1*1101+ haplotypes were significantly higher in asthmatics compared with atopic and non-atopic controls (19.6% vs 2.2% vs 2.3%, $PC < 0.05$). The HLA-DRB1*1101, DQA1*0501, DQB1*0301 haplotype was found significantly increased in the patients vs non-atopic controls (15.4 vs 1.1%, $PC < 0.01$). The serum levels of specific IgE were detectable in both atopic asthmatics and atopic controls; however, it was higher in atopic asthmatics vs atopic controls Der p (median, 58.7 vs 2.7 kU/L, $P < 0.001$) and Der f (median, 46.9 vs 2.7 kU/L,

$P < 0.001$). No linkage between MHC genes and mite-atopy could be documented on informative families with two or more atopic siblings.

Conclusions We have identified an association between the haplotype HLA-DRB1*1101, DQA1*0501, DQB1*0301 and atopic asthma that confers susceptibility to develop mite-sensitive asthma to atopics (relative risk, RR 8.2), and to non-atopic controls (RR = 15.8) that carry this haplotype. Conversely, the allele HLA-Cw7 was absent in the asthmatics studied and had higher frequencies in the atopic (RR = 0.05) and non-atopic (RR = 0.08) controls. Thus, it may have a protective role for developing atopic asthma in the population studied.

Keywords: atopy, asthma, HLA-class I alleles, HLA-class II alleles, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, IgE, house dust mite allergy, genetics, HLA-DRB1*11, eosinophils

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Introduction

Atopy is a genetic predisposition to respond to environmental allergens with immunoglobulin E (IgE) antibodies instead of IgG [1]. It has a spectrum of manifestation ranging from the asymptomatic individual that never develops clinical manifestations in any of the target organs (i.e. lung, skin and gut), to the more severe forms of this diathesis such as severe asthma and/or atopic dermatitis. Mite-sensitive asthma is highly frequent in Venezuela, and worldwide [2,3]. Asthma morbidity and mortality has been increasing in Latin America [4] and worldwide until the 1980s [5,6]. Despite a trend in the last decade of a gradual reduction in asthma mortality [7,8] the prevalence is still very high. Identification of susceptible populations at risk of developing atopic asthma is important for prevention strategies.

The genetics of IgE, atopy and atopic asthma are complex. The major histocompatibility complex (MHC) genes have been implicated in the genetic control of these phenotypes [9,10]. MHC class II antigens have been shown to restrict the antigen presentation of allergens to T cells [11,12]. Interestingly HLA-DRB1*0101, DPB1*0401, DPB1*0402 DPB1*0501 have been found *in vitro* to restrict peptides of the group I allergen of *D. pteronyssinus* (Der p 1) [11], whereas DRB1*1101 has been associated with *in vitro* restriction of group II of *D. pteronyssinus* (Der p 2) [12]. A previous study in a Chinese population found an association between a MHC-class II antigen, HLA-DQw2 and mite-reactivity in asthmatic children [13]. However, despite a number of different immunogenetic studies in mite-sensitive asthmatic patients and demonstration of linkage with MHC genes [14–16], no other associations have been reported to date. A study carried out in mulattos in Colombia found a decrease frequency of DPB1*0401 in mite-sensitive asthmatics compared with non-atopic controls, but found no associations with any other class II

alleles [17]. Comparisons between studies are difficult due to differences in ethnic groups and in the phenotypic definitions chosen for asthma and atopy in each particular study.

In this study, we present an immunogenetic family analysis of Venezuelan mite-sensitive asthmatics that includes: (1) linkage analysis, (2) sibpair analysis, and (3) association analysis with high resolution MHC-class I and MHC-class II haplotypes. Two control groups were used to ascertain association with atopy and/or asthma, atopic controls (asymptomatic, non-asthmatics) and non-atopic controls from the same geographical region and same ethnic background. We report the association of an HLA DR-DQ haplotype with susceptibility to mite-sensitive asthma.

Materials and methods

Study subjects and families

This study was approved by the Human Research Ethic Committee of the Military Hospital 'Carlos Arvelo'. The total sample tested consisted of 135 individuals, 71 belonging to 20 families or sibships (single parent with one or more siblings, or a group of at least two siblings without parents) and 64 unrelated subjects: 41 healthy non-atopics and 23 healthy atopics. Atopy to mite was defined as a positive skin prick test to *Dermatophagoides* spp. mix and/or detectable specific IgE to Der p and/or Der f. Mite atopic asthma with or without rhinitis was defined as the presence of a positive skin test to *Dermatophagoides* spp. and clinical diagnosis of asthma as defined by the American Thoracic Society criteria [18] with or without clinical rhinitis.

Atopic asthmatics

Twenty non-related mestizos with atopic asthma (age range 5–35 years; 14 females and six males) were enrolled from

the outpatient clinic of the Department of Allergy and Clinical Immunology of the Military Hospital 'Carlos Arvelo' in Caracas (Table 1). All patients had been diagnosed as having mild, moderate or severe asthma as defined by the National Asthma Education Program Expert Panel Report [19]. Inclusion criteria were: (1) at least 2 years of diagnosis of atopic asthma with or without rhinitis; (2) skin test positive to *Dermatophagoides* spp. (mixture of Der p and Der f); (3) two generations of Venezuelan-born ancestors (parents and grandparents); and (4) treatment only with β_2 -agonist or theophyllin as rescue medication. Exclusion criteria included: (1) concomitant systemic disease or atopic dermatitis; (2) smokers; and (3) regular treatment with inhaled, oral or systemic steroids.

Patients' blood was drawn during an asymptomatic period. Patients included in the study had to withhold theophylline for 7 days, antihistamines for 24 h, and β_2 -agonists for at least 6 h before blood collection.

Atopic families

Twenty families from the 20 index cases described above resided in Caracas and originated from different regions of Venezuela. Seventy-one individuals (31 parents and 40 siblings) were tested for HLA class I and class II haplotypes by serology and DNA oligotyping (see below). All members

of the families were skin-prick tested to ascertain atopic status and completed a health clinical interview to determine general health history including clinical atopy, i.e. asthma, rhinitis or atopic eczema.

Non-atopic controls

A total of 41 controls were included in the study to ascertain HLA gene frequencies in non-atopic, non-asthmatic subjects. Thirty-four unrelated healthy mestizos (8–36 years, 21 females and 13 males), and six unrelated parents of these non-atopic subjects (34–52 years, four females, and two males). Inclusion criteria comprised: (1) skin-prick negative test to a panel of 15 environmental allergens; (2) two generations of Venezuelan ancestors; and (3) absence of history of clinical atopy, i.e. asthma, rhinitis or atopic eczema or other systemic diseases. Exclusion criteria comprised: (1) systemic or chronic disease and (2) smokers.

Atopic controls

Twenty-three healthy mestizos (age range 18–52 years, two females, 21 males) were selected to ascertain HLA genes associated with mite atopy and not asthma. Inclusion criteria

Table 1. MHC class I and class II haplotypes of non-related asthmatic subjects (probands)¹

Clinical severity on asthma					Haplotype 1							Haplotype 2									
ID	Classification	A	B	C	DRB1	DRB3	DRB4	DRB5	DQA1	DQB1	DPB1	A	B	C	DRB1	DRB3	DRB4	DRB5	DQA1	DQB1	DPB1
7	Moderate	2	44	X	1101	02			0501	0301	1401	2	42	5	1303	0101			0501	0301	1001
13	Moderate	24	51	X	1101	02			0501	0301	0401	2	45	X	1102	02			0501	0301	0402
15.3	Mild	30	13	2	1101	02			0501	0301	0101	2	53	4	1101	02			0102	0602	1701
33	Mild	10	24	51	1101	02			0501	0301	0401	33	14	8	0102				0101	0501	0401
45	Moderate	24	44	2	1101	02			0501	0301	0402	24	14	8	1101	0301			0501	0504	0101
65	Severe	ND	ND	ND	1101	0301			0501	0301	0402	ND	ND	ND	1305	02			0501	0301	0101
16	Mild	24	18	5	1104	02			0501	0301	0401	24	7	X	1501			0101	0102	0602	0402
2	Mild	3	51	1	1401	02			0101	0503	0402	28	51	4	0700		0101		0201	0201	0401
21	Mild	28	35	4	0102				0101	0501	1101	31	61	X	0404		0101		03	0302	0401
25	Mild	24	93	6	0700		0101		0201	0201	1401	2	18	5	0301	02			0501	0201	0401
29	Moderate	2	21	X	0405		0101		03	0302	3401	2	61	3	0411		0101		03	0302	0402
35	Moderate	3	35	1	0103	0103			0101	0501	0401	24	35	4	0407		0101		03	0302	1401
39	Mild	30	42	X	0302	0101			0401	0401	0101	3	7	X	1501			0101	0102	0602	0401
48	Mild	3	7	X	0401		0101		03	0302	0301	33	14	8	0700		0101		0201	0201	1701
51	Mild	3	44	X	1304	0101			0103	0603	0401	34	14	8	0801				0401	0402	0301
54	Moderate	2	51	6	1401	0101			0501	0301	1401	2	42	X	0700		0101		0201	0201	0101
61	Moderate	1	17	5	1503			0101	0102	0602	0401	29	8	5	0301	0101			0501	0201	1701
63 ³	Moderate	1	5	4	ND				ND	ND	ND	24	8	X	0301	0101			0501	0201	0401
67	Severe	31	7	4	402/0	0101			0501	0201	1101	24	18	X	0301	02			0501	0201	0401
70	Severe	2	7	X	0404		0101		03	0302	0501	3	35	4	1503			0101	0102	0602	0401

¹All the patients had specific IgE for Der p and Der f and had history of rhinitis. ²Clinical classification according to the National Asthma Education Program Expert Panel Report [19]. ³Deduced class II haplotype from maternal haplotypes. DQA*03=0301/0302. ND, not done.

Table 2. MHC-class I allele frequencies in atopic patients with asthma as well as rhinitis and non-atopic controls

Allele	Asthmatic (n = 38)		Controls (n = 84)	
	+	%	+	%
<i>HLA-A alleles</i>				
1	2	5.3	5	6.0
2	10	26.3	20	23.8
3	6	15.8	3	3.6
11	0	0	3	3.6
23	0	0	3	3.6
24	10	26.3	13	15.5
26	0	0.0	3	3.6
28	1	2.6	6	7.1
29	1	2.6	6	7.1
30	3	7.9	8	9.5
31	2	5.3	6	7.1
32	0	0	2	2.4
33	2	5.3	2	2.4
34	1	2.6	1	1.2
68	0	0	2	2.4
w69	0	0	1	1.2
<i>HLA-B alleles</i>				
7	4	10.5	10	11.9
8	2	5.3	1	1.2
13	1	2.6	1	1.2
14	4	10.5	5	6.0
15	1	2.6	1	1.2
17	1	2.6	5	6.0
18	3	7.9	4	4.8
21	1	2.6	1	1.2
25	0	0.0	1	1.2
27	1	2.6	0	0
35	3	7.9	14	16.6
37	0	0.0	2	2.4
39	1	2.6	2	2.4
40	0	0.0	4	4.8
42	3	7.9	0	0.0
44	4	10.5	8	9.5
45	2	5.3	0	0
48	0	0.0	1	1.2
50	0	0.0	3	3.6
52	0	0.0	1	1.2
51	4	10.5	8	9.5
53	1	2.6	3	3.6
61	2	5.3	2	2.4
60	0	0.0	3	3.6
62	0	0.0	2	2.4
63	0	0.0	2	2.4

HLA-Cw alleles

1	2	5.3	3	3.6
2	2	5.3	1	1.2
3	1	2.6	8	9.5
4	5	13.2	16	19.0
5	5	13.2	2	2.4
6	2	5.3	8	9.5
8	4	10.5	8	9.5
7	0	0	12	14.3*
10	0	0	1	1.2
9	0	0	2	2.4
X	17	44.7	23	27.4

* $P=0.017$, $PC=0.19$.

were: (1) total IgE levels above 75 quartile from Venezuelan normal non-atopic controls (children + adults) >140.3 IU/mL; (2) presence of detectable levels of specific Ig E to Der p and/or Der f >0.35 kU/L; (3) two generations of Venezuelan ancestors; and (4) absence of clinical atopy i.e. asthma, rhinitis or atopic eczema or other systemic diseases. They were selected from a larger healthy control series that were part of an immunogenetic study of Venezuelan healthy population [20] for whom total IgE was determined (data not shown).

Haplotypes

HLA-A, C, B, DRB1, DQA1, DQB and DPB1 loci haplotypes were ascertained after analysis of the nuclear family pedigrees or parent-child pairs for asthmatics and non-atopic controls. For unrelated atopic controls, haplotypes were assigned on the basis of linkage disequilibria between HLA alleles and former Venezuelan family studies information [20].

The total number of haplotype for MHC-class I and class II for the asthmatics were 38 and 39, respectively (Table 1). The total number of healthy non-atopic haplotypes were 82 for MHC-class I haplotypes and 88 for MHC-class II, (frequencies shown in Tables 2 and 3), 68 and 74 (MHC class I and class II, respectively) from 41 non-related controls, and 14 MHC class I and class II non-disease haplotypes from parents of asthmatic families. The healthy atopic haplotypes were 24 for MHC-class I (data not shown) and 46 class II haplotypes (data not shown and Table 4).

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient [21]. Briefly, 20 cm^3 of whole blood were diluted 2:1 in phosphate-buffered saline (PBS) on a Ficoll-Hypaque gradient (density 1070 ± 0.001 g/mL)

Table 3. MHC class II allele frequencies in atopic patients with asthma as well as rhinitis and non-atopic controls.

Allele	Asthmatics (n = 39)		Controls (n = 88)	
	+	%	+	%
<i>HLA-DRB1 alleles</i>				
01	3	7.7	9	10.2
15	4	10.3	8	9.1
16	0	0	6	6.1
03	05	12.8	5	5.7
04	6	15.4	24	27.3
11	10	25.6*	4	4.5
13	3	7.7	11	12.5
14	3	7.7	3	3.4
07	4	10.3	12	13.6
08	1	2.6	2	2.3
09	0	0	3	3.4
10	0	0	1	1.1
<i>HLA-DQA1 alleles</i>				
0101	4	10.3	10	11.4
0102	6	15.4	15	17
0103	1	2.6	9	10.2
0201	4	10.3	11	12.5
0301/02	6	15.4	24	27.3
0401	2	5.2	3	3.4
0501	15	41**	16	18.2
<i>HLA-DQB1 alleles</i>				
0102	0	0	1	1.13
0201	9	23.1	18	20.5
0301	11	28.2	12	13.6
0302	6	15.4	21	23.9
0303	0	0	1	1.1
0401	1	2.6	0	0
0402	1	2.6	3	3.4
0501	3	7.7	11	12.5
0502	0	0	3	3.4
0503	1	2.6	0	0
0504	2	5.2	0	0
0601	0	0	2	2.3
0602	4	10.3	9	10.2
0603	1	2.6	6	6.8
0605	0	0	1	1.1
<i>HLA-DPB1 alleles</i>				
0101	5	12.8	10	11.9
0201	0	0	5	6
0202	0	0	4	4.8
0301	2	5.1	6	7.1
0401	11	28.2	23	27.4
0402	9	23.1	12	14.3

0501	1	2.6	1	1.2
0601	0	0	3	3.6
1001	1	2.6	4	4.8
1101	2	5.1	2	2.4
1301	0	0	2	2.4
1401	4	10.3	8	9.5
1501	0	0	2	2.4
1701	3	7.7	1	1.2
1801	0	0	1	1.2
3401	1	2.6	0	0

* $P = 0.0011$, $PC = 0.013$; ** $P = 0.0091$, $PC = 0.063$.

and centrifuged at 400 *g* for 30 min. The milky interface was recovered, washed twice and resuspended in PBS with 3% fetal calf serum (FCS) (Life Technology, Maryland, USA). Cells were cryopreserved until needed for HLA serology typing.

HLA serology

HLA-A, C and B allele typing was performed by the microcytotoxicity method [22] using enriched T cell suspensions from the cryopreserved PBMC and sera from the 11th International Histocompatibility Workshop [23].

DNA isolation

DNA isolation was done from acid citrate dextrose (ACD) preserved blood by a quick lysis method [24] or by a salting-out method with minor modifications [25].

Polymerase chain reaction amplification

DNA samples were amplified by polymerase chain reaction (PCR) for DRB generic, DQA1 and DQB1 loci in a 100 μ L reaction mixture containing 50 pmol/L of each primer, 50 mmol/L KCL, 10 mmol/L TRIS-HCL pH 8.3, 200 mmol/L of each deoxynucleotide, 2.5 U of Taq polymerase (AmpliTaq DNA polymerase, Perkin-Elmer Cetus, Norwalk, CT, USA or Taq polymerase, Promega Corporation, Madison, WI, USA) and 1.5–2 mmol/L $MgCl_2$. The sequence of the primers and conditions for amplification used in this study are available elsewhere [26–29].

In addition, allele specific amplification for DRB1*04, DRB1*15/16, DRB1*03, DRB1*11, DRB1*12, DRB1*13, DRB1*14 and DRB1*08 were carried out when required. The primers were identical to those described in the 11th International Histocompatibility workshop reference protocol [30]. For DRB1*03, DRB1*11, DRB1*12, DRB1*13, DRB1*14 and DRB1*08 allele high resolution typing, identical amplification conditions were used as for generic DRB amplification [29]. For DRB1*15/16 and

DRB1*04 allele amplification (MgCl_2 at 2 mmol/L), an initial denaturing step at 94 °C was followed by 30 cycles (35 for DRB1*04) of denaturing at 96 °C for 10 s, annealing at 61 °C for 45 s (60 °C for DRB1*04), an extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min were carried out in a Perkin-Elmer 9600 Thermal cycler (Norwalk, CT, USA). Negative controls (no DNA) were always included to detect contamination (distilled water). Amplification was confirmed by electrophoretic analysis of 5 μL of PCR amplified product in a 1XTBE-2% Nusieve-1% Seakem agarose (FMC Bioproducts, Rockland, ME, USA) gel at 150 V for 1 h, and staining with ethidium bromide. Dot blot prehybridization and hybridization conditions have been described elsewhere [26,27,29].

DRB, DQA1 and DQB1 allele assignment

The DQA1 and DQB1 alleles were determined in the locus-specific PCR-amplified products by sequence-specific oligonucleotide probe hybridization (PCR-SSO) as described elsewhere [27,28]. For DRB generic and allele-specific typing (DRB1*04, DRB1*15/16, DRB1*03, DRB1*11, DRB1*12, DRB1*13, DRB1*14 and DRB1*08) a panel of 36 sequence specific oligonucleotide probes were used. The SSO probes used have been described in the reference protocol of the 11th International Histocompatibility Workshop [30] (data not shown). The sequence, orientation, codons, allele specificity and melting profile of those SSO probes used in this study but not described on the 11th International Histocompatibility Workshop were designed based on published MHC-class II nucleotide sequence and were tested against an extensive panel of homozygous and heterozygous cell panels [29].

Linkage analysis

To determine formally whether the presence of atopy (defined as the presence of positive prick test for *Dermatophagoides* spp. and/or detectable levels of specific IgE to Der p and Der f) was linked to MHC haplotypes, linkage analysis was carried out between atopy and MHC haplotypes using the logarithm of the odds (LOD) score [31,32]. LOD scores for each family were added and the recombination fraction with highest value (Z) was the maximum likelihood estimate. Linkage would be indicated if the sum was greater than three for a certain Z which was <0.05 .

Skin-prick test

Skin-prick tests were performed with a panel of common aeroallergen extracts (glycerol diluent) of the Caracas (Venezuela) area: Red top, Grass mix (*Cinodon dactylon*, *Dactylis glomerata*, Orchard and Timothy), ragweed g./

Ambrosia, dust mix, *Dermatophagoides* spp. (Der p and Der f), Fungui mix (*Hormodendrum*, *Alternaria temis*, *Aspergillus fumigatus*, *Cladosporium* and *Penicillium*), dog epithelium, cat pelt, cow epithelium, cotton, wool, kapok, piretro, feathers, (Hollister-Stier/Miles laboratories and Bioalergen laboratories, Universidad Central de Venezuela, UCV, Venezuela). Glycerol diluent and histamine were used as the negative and positive controls, respectively. A positive skin response was read at 15 min as a weal 3 mm above the negative control of the longest and orthogonal diameter.

Serum-specific and total IgE

Serum-specific IgE was quantified by the CAP system radioimmunoallergosorbent (RAST) for specific IgE to Der p and Der f. (Pharmacia Diagnostics, NJ, USA). The lower level of detection was 0.35 kU/L and the maximum was 100 kU/L. The levels of serum-specific IgE for Der p and Der f are expressed in kU/L. Based on Pharmacia standard values levels of Der p and Der f <0.35 considered absent or undetectable, 0.35–0.7 kU/L are considered low levels, 0.7–3.5 kU/L moderate, 3.5–17.5 kU/L high and above 17.5 kU/L very high levels. Total serum levels of IgE were quantified by means of a commercial enzyme-linked immunosorbent assay kit (Abbot laboratories, IL, USA) following manufacturer's instruction. Values are expressed in International Units/mL (IU/mL) (one IU = 2.4 ng). The lower detection level is of 8 IU/mL and the maximum level was 25 000 IU/mL.

Statistical analyses

HLA-A, B, C, DRB1, DQA1, DQB1 and DPB1 allele frequencies and three-locus haplotype (HLA-DRB1, DQA1 and DQB1) frequencies were determined by direct counting.

Comparison of alleles and haplotype frequencies were made by the Fisher exact test. Chi-square was used for sibpair analysis. *P*-values were corrected for the number of comparisons (*PC*) and were considered significant when ≤ 0.05 . The segregation analysis was done separately in families with affected and non-affected parents. The Mann–Whitney Rank Sum test was used for unpaired data and the Kruskal–Wallis ANOVA rank test with Dunn's method for multiple comparisons. Sigma Stat (Jandel Scientific/SPSS Science) statistical software was used for all the statistical analyses.

Relative risk

The relative risk (RR) estimates how many times more the carrier of a specific allele or haplotype was likely to have a

specific disease associated with the particular genetic marker studied. It was calculated according to the formula $RR = a \times d / b \times c$, where a was the number of patients positive for the allele or haplotype; b was the number of patients negative for the allele or haplotype; c was the number of controls positive for the allele or haplotype and d was the number of controls negative for the allele or haplotype. Haldene's modification was used in sets containing 0 [33].

Results

Clinical characteristics of subjects and families

Twenty asthmatics were enrolled from the outpatient clinic of the Department of Allergy and Clinical Immunology of Hospital Carlos Arvelo. They met all inclusion and exclusion criteria and served as index cases for the selection of the asthmatic families and also served as a series of non-related asthmatics (Table 1) to perform the association study. All asthmatic patients had positive skin-prick tests to *Dermatophagoides* spp. and also had history of concomitant rhinitis at some point in their course of their atopic disease. Patients were clinically asymptomatic the day of the blood collection. All had detectable levels of specific IgE (Fig. 1). Only five out of 20 patients had, in addition to their mite sensitivity, positive prick tests to other allergens as determined by the prick test done with a panel of 14 environmental allergens. Patient #15.3 was allergic also to dog epithelium, cat pelt and bovine fur and to a fungi mix;

#16 was also allergic to Red Top pollen, fungi mix and wool, #29 to feather and bovine fur, #39 to cotton and #54 to dog epithelium. All patients had asthma and rhinitis onset during childhood. Sixteen families had at least one affected parent and four families had two healthy parents, but had affected second degree relatives (uncles or aunts).

Association analysis

Table 1 shows the MHC class I and II haplotypes of 20 non-related asthmatics subjects (proband). Thirty-eight HLA class I and 39 HLA class II haplotypes were determined in the 20 atopic asthmatics studied. The paternal haplotype corresponded generally to haplotype #1 and the maternal haplotype to haplotype #2 with the exception of the HLA-DRB*11-positive haplotypes that were arbitrarily assigned into haplotype #1 for depiction reasons. The asthma severity was not associated with a specific haplotype. Twenty-four HLA class I and 46 HLA class II haplotypes were determined in the atopic control group (data not shown and Table 4) and 84 HLA class I and 88 HLA class II in the non-atopic control group (frequencies shown in Tables 2–4).

Among the class I allele frequencies there were no differences between asthmatics and non-atopic controls (Table 2) or between asthmatics and atopic controls (data not shown). However, it is noteworthy that HLA-Cw7 was completely absent among the asthmatics, whereas its frequency was higher among atopic controls (20.8%, $RR = 0.051$, $P = 0.006$, $PC = 0.066$) and non-atopics (14.3%, $RR = 0.075$, $P = 0.017$, $PC = 0.19$) compared with asthmatics (0%). This difference did not reach significance when corrected for the number of HLA-Cw alleles tested. The HLA class I allele and haplotype frequencies for the atopic controls were non-significantly different from the non-atopic controls (data not shown).

Comparison of class II allele frequencies demonstrated a significant association between the HLA-DRB1*11 allele in atopic asthmatics relative to non-atopic controls (25.6 vs 4.5%, $RR = 5.6$, $P = 0.0011$, $PC = 0.013$) (Table 3). Also a marginal association was found with DQA1*0501 (41% vs 18.2%, $RR = 2.3$, $P < 0.0091$, $PC = 0.063$). No other associations were found among the DQB1 and DPB1 allele. To ascertain whether the HLA-DRB1*11 allele was associated with atopy or asthma, the HLA-class II gene frequencies of the atopic controls vs the non-atopic controls were compared (data not shown). There were non-significant differences among the atopic and non-atopic controls, however, the frequency of HLA-DRB1*11 allele was higher among the atopics (15.2%, $RR = 3.3$, $P = 0.07$) relative to the non-atopic controls (4.5%). A gradient effect could be observed where the asthmatics had 25.6%, the atopic controls 15.2% and the non-atopic controls 4.5%.

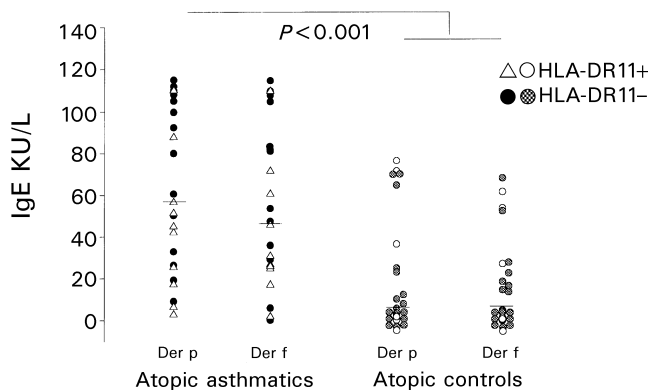


Fig. 1. Specific IgE levels in serum of atopic asthmatics and atopic controls. Levels of specific IgE for Der p and Der f are represented by the dots and triangles in KU/L (Y-axis). Median values are the transverse line in each set of samples. The probability value was obtained by comparing serum levels of Der p and Der f of atopic asthmatics ($n = 24$ and $n = 22$, respectively) vs. atopic control serum levels of Der p and Der f ($n = 23$), respectively. Empty dots and triangles represent HLA-DRB1*11 positive subjects and black and gray dots represent HLA-DRB11- asthmatics and atopic controls, respectively.

We extended our analysis to specific haplotypes involved in this association and by performing high-resolution analysis, we determined that the asthmogenic haplotypes carried HLA-DRB1*1101 (Table 4), whereas the atopic controls had mainly other HLA-DRB1*11 alleles. Table 4 shows all the HLA-DRB1*11+ haplotypes found among asthmatics and the two control groups. There was a significant association between the HLA-DRB1*1101,DQA1*0501,DQB1*0301 haplotype and atopic asthma (15.4%) compared with non-atopic controls (1.1%, $RR = 15.8$, $P = 0.0028$, $PC = 0.008$) and a trend compared with atopic controls (2.2%, $RR = 8.2$, $P = 0.034$, $PC = 0.09$). Furthermore, when all the DRB1*1101+ haplotypes were added, the percentage was significantly higher among atopic asthmatics (19.6%) compared with both control groups; atopic (2.2%, $RR = 11.6$, $P = 0.007$, $PC = 0.021$) and non-atopics controls (2.3%, $RR = 11.1$, $P = 0.001$, $PC = 0.003$) (Table 4).

HLA-DRB1*03 alleles were higher in the asthmatics compared with the atopic controls (12.8% vs 0%, $P = 0.018$, $PC = 0.21$) but this difference was not statistically significant.

Sibpair analysis

The segregation of HLA haplotypes in families grouped according to the presence of affected or non-affected parents showed random segregation 1:2:1 in healthy and affected siblings (data not shown).

Linkage analysis

There were 11 informative families with an atopic parent and a minimum of two children with atopy to Der p or Der f with or without asthma and/or rhinitis. The number of recombinants were those children with positive atopy with discordant paternal or maternal haplotype or healthy with concordant haplotype. The LOD (Z) was against linkage between MHC and atopy to Der p and Der f. However, four of the 11 informative families had no recombinants with a total Z of 1.32 at 0.05 recombination fraction.

Specific IgE levels

To determine whether the levels of IgE could distinguish among the atopic asthmatic and the atopic non-asthmatic

Table 4. Polymorphism of HLA-DRB1*11 DQA1*DQB1* haplotypes among atopic asthmatic atopic and non-atopic controls

DRB1*	DQA1*	DQB1*	Atopic asthmatics (n=39)		Atopic controls (n=46)		Non-atopics controls (n=88)	
			+	%	+	%	+	%
1101	0501	0301	6	15.4 ^{*,**}	1	2.2	1	1.1
	0102	0602	1	2.6	0	0.0	1	1.1
	0102	0504	1	2.6	0	0.0	0	0.0
		Total	8	19.6 ^{†,‡}	1	2.2	2	2.3
1102	0301	0503	1	2.6	1	2.2	0	0.0
	0501	0301	0	0	0	0.0	1	1.1
1104	0501	0301	1	2.6	1	2.2	1	1.1
1106	0501	0301	0	0	1	2.2	0	0.0
1107	0501	0301	0	0	1	2.2	0	0.0
1110	0501	0301	0	0	1	2.2	0	0.0
1100	01	0602	0	0	1	2.2	0	0.0

* $P = 0.0028$, ($PC = 0.008$) atopic asthmatics vs. non-atopic controls, $RR = 15.8$; ** $P = 0.03$, ($PC = 0.09$) atopic asthmatics vs. atopic controls, $RR = 8.2$; [†] $P = 0.001$, ($PC = 0.003$) atopic asthmatics vs. non-atopic controls, $RR = 11.1$; [‡] $P = 0.007$, ($PC = 0.021$) atopic asthmatics vs. atopic controls, $RR = 11.6$.

phenotype, the levels of specific IgE for Der p or Der f were measured in asthmatic probands ($n = 20$) their siblings with atopy and asthma ($n = 4$) and the atopic controls ($n = 23$). The serum levels of specific IgE were detectable in both atopic asthmatics and atopic controls, however, the levels were higher in atopic asthmatics vs atopic controls Der p-specific IgE (median, 58.65 vs 2.64 kU/L, $P < 0.001$) and Der f-specific IgE (median, 46.9 vs 1.62 kU/L, $P < 0.001$). Since an association had been found between the HLA-DRB1*11 allele and atopic asthma, this allele had a relative high frequency in the atopic controls group (see above), it was important to distinguish the HLA-DRB11+ individuals among the atopic asthmatics and atopic controls. In Fig. 1, empty triangles and circles depict the IgE levels of the HLA-DRB11+ atopic asthmatics and atopic controls, respectively. It is noteworthy to emphasize, that in the asthmatic group 6/7 HLA-DRB11+ patients were HLA-DRB1*1101 as compared with 1/6 HLA-DRB11+ subjects in the atopic control group, ($P = 0.029$) (see Tables 1 and 4). Importantly, differences among the specific IgE levels of the HLA-DRB1*11+ and HLA-DRB1*11- atopic asthmatics and among the specific IgE levels among the HLA-DRB1*11+ and HLA-DRB1*11- atopic controls were not significant. Of interest, 3/6 HLA-DRB1*11+ atopic controls were among the subjects with higher levels of specific IgE.

All non-atopic controls, by definition, had non-detectable levels of specific IgE. This was documented by negative prick tests to a panel of 14 environmental allergens (see methods). Der p serum-specific IgE was measured in a subgroup of 17 non-atopic subjects (including four non-atopic sibling from the asthmatic families), and serum Der f-specific IgE was measured in five non-atopic subjects (including three siblings). As expected, levels of specific IgE for either species of mite were not detectable (data not shown). The margin of detection of the specific IgE assay was 0.35 kU/L, and for statistical comparison an arbitrary value of 0.3 was assigned to all subjects with values < 0.35 kU/L. There was a significant difference between the specific IgE levels of both mites species between atopic asthmatics and non-atopic controls ($P < 0.0001$) but the difference between the atopic and non-atopic controls did not reach statistical significance ($P = 0.063$) (data not shown). In general, atopic asthmatics had higher levels of specific IgE for Der p and Der f than the atopic controls, although there was some overlap, suggesting that there are other factors needed to define the asthmatic phenotype.

Discussion

Our findings are the first reported evidence of an association between HLA-DRB1*1101, DQA1*0501, DQB1*0301 haplotype and mite-sensitive asthma.

There was a gradient effect in the frequency of HLA-DRB1*11 allele with higher frequency in asthmatics, followed by the atopic controls and non-atopic controls (25.6, 17.4 and 4.2%, respectively) suggesting a possible role for HLA-DRB1*11 in atopy susceptibility with or without asthma. The frequency in our non-atopic controls is consistent with the published frequency of HLA-DRB1*11 in a healthy population in a larger cohort [20].

More important, the high-resolution analysis of HLA-DRB1*11 allowed us to identify the HLA-DRB1*1101, DQA1*0501, DQB1*0301 as the asthmogenic haplotype (Table 4). These results are important because *Dermatophagoides* spp. antigens have been shown to be restricted by HLA-DR and DQ antigens and interestingly there are some epitopes of the group 2 allergen of Der p that are presented by HLA-DRB1*1101 [12]. The HLA-DRB1*1101 has been shown to have a substitution in residue 86 of a Glycine (G86), whereas for example HLA-DRB1*1104 has a Valine (V86) [34]. This change in residue could determine variability in the recognition of peptides by T cells, whether being presented by one molecule or the other. This has been shown with tetanus toxoid peptides [34]. It could be speculated that a conserved region on the HLA-DRB1*11 molecule is critical for a shared Der p and Der f epitopes, but that the HLA-DRB1*1101 may have differential binding activity to the *Dermatophagoides* peptides implicated in an amplified allergic TH2 cell-mediated response which could influence asthma pathogenesis. Further *in vitro* studies testing the T-cell cytokine responses induced by the different APC bearing different HLA-DRB1*11 alleles would be needed to test this hypothesis.

It is known that Interleukin-4 (IL-4) and Interleukin-5 (IL-5) are produced by TH2-like cells and are required for IgE isotype switching and differentiation and maturation of eosinophils, respectively [35–37]. Interestingly, asthmatics showed higher levels of specific IgE as compared with the atopic controls (Fig. 1) similar to the report in asthmatics allergic to ragweed compared with atopics that only had rhinitis [38]. Eosinophils are important mediators of the allergic lung inflammation and often eosinophilia (increased number of eosinophils in peripheral blood) is found in atopic and non-atopic asthmatics [39]. In our sample, only asthmatics showed higher levels of eosinophil cell counts (median, 640 cells/mm³, $P < 0.001$) relative to atopic (110 cells/mm³) and non-atopic (160 cells/mm³) controls, suggesting that activation of an alternative inflammatory pathway other than allergen-specific IgE-mediated inflammation is needed to develop an atopic asthma phenotype. This finding is consistent with previous reports between eosinophilia and the asthma phenotype [40,41]. Importantly, the asthmatics studied were in a symptom free period, thus, high total eosinophil counts do not seem to be associated with asthma 'activity' but with a probable underlying

disregulation of the immune response that is reflected in the number of eosinophils found in peripheral blood. Previous studies support this concept, since the correlation between the degree of airflow obstruction, disease severity and the number of activated eosinophils is stronger than the correlation with total number of eosinophils [41,42]. Taken together, higher levels of IgE and eosinophilia would suggest an amplified TH2 response in the atopic asthmatics compared with the atopic controls.

Linkage analysis of 11 informative pedigrees with HLA and specific IgE to *Dermatophagoides* antigens showed no evidence for linkage. However, four families with no evidence of recombinants and a LOD Z of 1.32 at 0.5 recombination suggested genetic heterogeneity. These results are consistent with the concept that more than one gene is involved in the pathogenesis of atopy as shown in atopy to ragweed [43]. The possibility of type II error cannot be discarded given the small number of informative families. Thus, a linkage analysis needs to be done in a larger number of families with atopy to *Dermatophagoides* spp.

The HLA-DRB1*03 frequency among asthmatics was comparable with the HLA-DRB1*03 among non-atopic controls. However, HLA-DRB1*03 antigen among atopic controls was totally absent, and it could be speculated that being atopic and HLA-DRB1*03+ makes a highly susceptible combination to become asthmatic. A previous study in ragweed-sensitive atopy found that HLA-DRB1*03 haplotypes were over-represented in atopic patients with rhinitis without asthma whereas HLA-DRB1*02, was more frequent in the atopics with rhinitis and asthma [39]. These differences between mite-sensitive and ragweed-sensitive asthma could be due to differential binding to HLA-DR antigens by each of the allergen peptides. Therefore, it is reasonable to speculate that *Dermatophagoides* peptides may be preferentially presented to T cells by HLA-DRB1*1101 and HLA-DRB1*03 alleles.

Previous HLA studies in other Latin American and Caucasian populations in mite-sensitive asthma have been published [14,16,17,44,45], however, none of these studies have found an association with the haplotype HLA-DRB1*1101,DQA1*0501,DQB1*0301.

In a study performed on Colombian asthmatics [17], a lack of association between HLA-DR-DQA-DQB haplotype and mite-sensitive asthma was reported. However, the researchers found a decrease in the frequency of the HLA-DPB1*0401. In our sample, the HLA-DPB1*0401 was comparable among asthmatics and non-atopic controls (Table 3) and data was not available for the atopic controls. This difference could be due to variable ethnic composition of the sample, since Caraballo and co-workers studied a mulatto population with a larger African admixture [17], whereas our sample was a mestizo population with a lower proportion of African admixture. There is evidence of different degrees of admixtures of

Caucasian, Amerindian and African genes among Latin American populations and thus of HLA gene frequencies. The HLA gene frequencies may differ from country to country or within countries in the region. This emphasizes that it is not appropriate to categorize study populations as 'Hispanic' or 'Latin American'.

A British study [16] failed to find a positive association between HLA markers and mite-sensitive atopy. There are two differences with our study which probably explain the difference with our findings: one is the ethnic origin of the subjects tested, and the second is that in contrast with our study, they included subjects in their association analysis that had Der p or Der f skin test reactivity with or without asthma, rhinitis or eczema. Another study performed in a French population found an association of atopy and HLA-DR4 and DR7, but again, this is a population of different ethnicity and there are differences in the definition of the phenotype of atopy, since they mixed mite and non-mite atopy with or without asthma-related symptoms [45]. In our study, we separated the three study groups, subjects with mite-sensitive asthma and rhinitis, a second group of asymptomatic atopics with specific IgE who had no clinical history of asthma, rhinitis or eczema and a third group of well-characterized non-atopics. Another important difference with the European studies is that in their atopic phenotype definition, in addition to the presence of specific IgE, they included a group of atopics based on increased levels of IgE. This raises an important difference between the diagnosis of atopy in tropical and non-tropical countries. The sole increase of total IgE without increase levels of specific IgE cannot be considered atopy in tropical countries where IgE can be elevated by parasite infection [3,46]. Given the complexity of phenotypes of atopy, atopic asthma and/or rhinitis, we believe that it is important to separate complex phenotypes for genetic analysis such as total IgE levels with atopy vs total IgE levels without atopy and vs the different atopic phenotypes, i.e. presence of allergen-specific IgE without disease or allergen-specific IgE with asthma and/or rhinitis and/or eczema [9].

An important aspect of the genetics of atopic asthma, is that genotypic vulnerability does not necessarily lead to the development of the disease. The low concordance among atopic monozygotic twin pairs emphasizes the significant role played by environmental factors in the manifestation of the disease [47]. In addition to environmental factors such as allergens, viral infections, cigarette smoking and air pollutants, there is increasing evidence for the role of emotional stressors as triggers or as risk factors for the development of asthma [48,49]. We agree with Sandford and co-workers [10] that the most likely benefit of identifying genes related to atopy, asthma or both is the possibility of enhancing our power of screening and in that way detect populations at risk that could take advantage of prevention interventions.

Previous studies suggest that preventing exposure to cigarette smoke and highly allergenic proteins in the first few years of life [50] can modify the clinical onset of atopic diseases. In addition, psychological stress can be another risk factor for the development of asthma in children with genetic risk. A prospective study in Colorado [48,49] showed that children born from asthmatic mothers with parental difficulties in a high stress family environment had higher incidence of asthma development at 3 years of age, compared with children from asthmatic mothers, in a low stress family environment and with non-parental problems. Thus, it could be possible to develop multidisciplinary preventive strategies, including environmental, nutritional and psychological components, to protect children early in life who are genetically at risk from developing atopic asthma.

In summary, we have identified HLA-DRB1*11 as a genetic marker for susceptibility to develop atopy to *Dermatophagoides* spp. in the Venezuelan population. The haplotype HLA-DRB1*1101, DQA1*0501, DQB1*0301 was specifically associated with susceptibility to atopic asthma, conferring an atopic individual an 8.2-fold greater chance of becoming asthmatic compared with a non-carrier of this haplotype (Table 4). Conversely, HLA-Cw7 may have a protective role for the development of atopic asthma since it is completely absent from the asthmatic subjects (Table 2). It is noteworthy that HLA-Cw7 is an allele frequently found in Amerindians [51,52] where asthma is of very low frequency compared with other populations of Venezuela [53]. These results have major implications in the Venezuelan population and, in general, to any population with a high incidence of HLA-DR11 alleles, such as several Caucasian populations [54].

The identification of the genetic markers of atopy and atopic asthma is of great value in identifying populations at risk that could be assisted with appropriate multidisciplinary prevention strategies.

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