

Immediate hypersensitivity to *Parthenium hysterophorus*. I. Association of HLA antigens and *Parthenium* rhinitis

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Summary

Lymphocytes collected from rhinitis subjects with strong positive skin reactions to the pollen allergens of *Parthenium hysterophorus* (American feverfew) having moderate to high titres of *Parthenium*-specific serum IgE were analysed for association of HLA-antigens covering 13 specificities of HLA-A, 17 specificities of HLA-B and eight specificities of HLA-DR loci by the NIH two-stage microlymphocytotoxicity assay. Comparison of the phenotypic frequencies of HLA-A and B antigens between *Parthenium* rhinitis subjects ($n=22$) and control subjects ($n=137$) did not suggest any significant association when tested for these antigen specificities. A significant correlation in the association of HLA-DR3 antigen with a relative risk of 11.33, however, was observed in *Parthenium* rhinitis subjects ($n=30$) when compared to controls ($n=50$).

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Introduction

HLA studies on families with individuals allergic to *Dermatophagoides farinae*, suggested that manifestation of allergic symptoms and IgE production are possibly regulated in an autorecessive HLA linked manner [1]. Initial studies performed on closely related families and unrelated patients suffering from hayfever suggested a HLA-associated responsiveness to various crude allergens [2]. A significant association between particular HLA types and specific IgE and IgG antibody responses to several crude and purified allergens have been reported [2,3].

Among the ragweed-sensitive Caucasian subjects who had elevated levels of IgE antibodies to Amb V (Ra5) following natural pollen exposure, 95% of them had an association with HLA-DR2 [4,5]. In addition, a significant association was observed between HLA-DR3 and ragweed (Ra5)-sensitive rhinitis patients and also between HLA-DR2 and IgE responders to RA5 suffering from both asthma and rhinitis [6]. A non-parametric

statistical analysis of the association of HLA-antigens with specific IgE responsiveness towards three highly purified rye pollen allergens (*Lol p* I, II and III) in Caucasian subjects revealed a significant association with HLA-DR3 antigens [7].

A high incidence of allergic rhinitis attributable to the pollen of *Parthenium hysterophorus* was observed in Bangalore and other parts of India where the weed is growing wild [8,9]. In view of the extensive crossreactivity between *Parthenium* and ragweed pollen allergens [10–12] and the association of the latter with HLA-DR2/Dw2 and DR3 [4–6], an attempt was made to study the association of IgE responsiveness to *Parthenium* pollen and HLA-A, B and DR antigens in rhinitic subjects with positive skin reactions to *Parthenium* pollen allergens.

Materials and methods

Material

Complete and incomplete Freund's adjuvant from Difco Laboratories (Detroit, U.S.A.), RPMI-1640 with L-glutamine and foetal calf serum were obtained from Gibco Laboratories (Great Island, U.S.A.). HLA-DR antisera were purchased from Behringwerke AG, (Marburg,

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FRG), and HLA-A and B antisera were a gift from the NIH Serum Bank (Bethesda, U.S.A.). Eosin, Y and formaldehyde were purchased from E. Merck, India (Bombay, India) and liquid paraffin from Sipali Chemicals (Madras, India). Ficol-hypaque was obtained from Pharmacia (Uppsala, Sweden) and membrane filters from Millipore (Bedford, U.S.A.). Terasaki 60-well microtest plates and repeating dispensers (PB-600) were purchased from NUNC (Raskild, Denmark) and Hamilton (Reno, U.S.A.) respectively. Heparin and trypan blue were from Sigma (St Louis, U.S.A.). Other reagents and materials were of analytical grade and available commercially.

Subjects

Normal subjects ($n=137$) and 22 rhinitis patients with positive skin-prick reactions to *Parthenium* pollen extract and elevated levels of *Parthenium*-specific serum IgE were selected to study the presence of HLA-A and B antigens. Another group of 37 *Parthenium*-sensitive subjects with elevated levels of specific IgE and 50 normal subjects were tested for their association with HLA-DR antigens. Some of the subjects were, however, common in both the groups.

Preparation of allergen extracts for skin testing

Defatted *Parthenium* pollen was extracted with phosphate-buffered saline (PBS), pH 7.4 for 20 hr at 4°C (1:10, w/v). The extract was then filtered, centrifuged at 20 000 *g* for 20 min and diluted with equal volumes of glycerol after passing through a sterile Millipore filter (0.45 μ m). The resultant allergen extract (1:20, w/v) stored at 4°C was used for skin testing.

Skin test procedure

Skin-prick tests were performed with extracts of *Parthenium* pollen (1:20, w/v) on the volar surface of the forearm of rhinitis subjects and controls. A 50% glycerine solution in PBS (pH 7.4) and histamine hydrochloride (1 mg/ml) dissolved in PBS-glycerol (1:1 v/v) served as negative and positive controls, respectively. The size of the weal reaction after 15 min was determined by measuring the diameter in two perpendicular directions and then halving the sum. The reaction was designated as positive if the net weal diameter was greater than 3 mm above the negative control.

Measurement of *Parthenium*-specific IgE antibodies

Parthenium pollen-specific IgE in the sera of *Parthenium*-sensitive rhinitis subjects and normal subjects was mea-

sured by microtitre RAST [13,14], using *Parthenium* allergen discs and Phadebas-RAST components including buffer solution and ^{125} I-labelled anti-human IgE obtained from Pharmacia (Uppsala, Sweden). The results are expressed as Phadebas RAST units/ml (PRU/ml) using Phadebas reference reagents [13].

HLA-antisera

Lyophilized powders of HLA-A, B and HLA-DR sera were reconstituted with sterile deionized triple-distilled water and stored in 100 μ l aliquots at -80°C until use. Whenever needed, one aliquot was taken and thawed at 4°C, centrifuged at 20 000 *g* for 10 min and used for dispensing into microtest plates. Thirteen specificities of HLA-A locus (A1, A2, A3, A9, Aw24, A10, A11, A19, Aw21, Aw19, A28, Aw31, Aw32 and Aw 33), 17 specificities of HLA-B locus (B5, B7, B8, B12, B13, B14, B15, Bw16, B17, Bw21, Bw22, B27, Bw35 Bw41, and Bw51) and six specificities of HLA-DR locus (DR1, DR2, DR3, DR4, DR5 and DR7) were used for HLA typing. In addition, the DR tray included antisera, defining 'super-typic' specificities DRw52 and DRw53.

Human Ab serum

Thirty millilitres of venous blood were collected from each of six AB Rh+ healthy male donors in a sterile tube and were allowed to clot. Serum was separated and centrifuged at 1000 r.p.m. for 15 min and inactivated at 56°C for 30 min. Aliquots (100 μ l) were then stored at -80°C and used as negative controls for HLA-typing.

Rabbit anti-human lymphocyte serum

Anti-human lymphocyte serum (ALS) was prepared by the method described by Gupta [15] and stored -80°C in 1 ml aliquots until use. ALS was diluted 1:2 and used as a positive control in HLA typing.

Rabbit complement

Sera of six healthy male rabbits without non-specific cytotoxic activity against normal human lymphocytes were pooled, aliquoted, and stored at -80°C. Samples of this normal rabbit serum, thawed and kept at 4°C for up to 30 min, were used as a source of complement.

Typing trays for cell panel establishment

A sterile kit of 83 typing sera comprising 30 specificities of HLA-A and B loci and 18 sera comprising eight specificities of HLA-DR loci were used. ALS and human AB serum/foetal calf serum, served as positive and negative

controls, respectively. A tray format was prepared according to the NIH specifications and 3–5 μ l of light liquid paraffin were added to each of the 60 wells of the microtest plate. One microliter of each typing serum was then dispensed into the appropriate well using a PB-600 Hamilton repeating dispenser with a 50 μ l syringe. The trays were then stored at $-80^{\circ}\text{C}/90^{\circ}\text{C}$ until use.

Isolation of peripheral blood lymphocytes and B-lymphocytes

Peripheral blood lymphocytes (PBL) were isolated as described previously [16]. Cells with a viability of more than 90% were selected for the study. B-lymphocytes were isolated from the peripheral blood lymphocytes by separation on a nylon fibre column as described by Werner *et al.* [17]. The concentration of B-cells was adjusted to 2×10^6 cells/ml and used in HLA typing for the DR loci.

HLA-typing

The NIH two-stage microlymphocytotoxicity test described by Mittal [18] was employed for HLA typing. The results were read under an inverted-phase contrast microscope and recorded according to the NIH reading scale.

Statistical analysis

Phenotype frequency (PF) was obtained by dividing the number of samples positive for a given analysis (n) with the total number of samples studied (N). Relative risk was calculated from 2×2 table by the method given by Svejgaard *et al.* [19]. Chi-square analysis with Yates' correction for antigen versus serum was calculated using the following formula:

$$\chi^2_y = \frac{[(ad - bc) - 0.5 N]^2 N}{(a + b)(c + d)(a + c)(b + d)}$$

Where N is the total number of samples and a, b, c, d are as indicated in a 2×2 table.

Results

For the cell panel establishment, the NIH starter kit comprising 30 specificities of HLA-A and B loci were used. The positive control (ALS) gave a strong reaction (8+) while the negative control (FCS/AB human serum) gave a 1+ reaction which was subtracted from the test wells while assigning the HLA-type. The PFs of HLA-A

Table 1. Frequencies of HLA-A and B antigens in *Parthenium*-sensitive rhinitis subjects and normal controls

HLA antigen	Subjects ($n=22$) (%)	Controls ($n=137$) (%)	Relative risk	χ^2_y
A1	45.45	32.12	1.76	0.97
A2	31.81	30.66	1.06	0.02
A3	13.64	12.41	1.11	0.04
A9	31.81	13.13	3.09	3.68
A10	18.18	8.76	2.31	0.96
A11	31.81	18.98	1.99	1.20
Aw19	4.55	17.52	0.22	3.49
Aw24	4.55	2.19	2.13	0.01
A28	4.55	5.11	0.88	0.41
B5	13.64	28.47	0.40	2.98
B7	27.27	11.68	2.84	2.67
B8	9.09	2.92	3.32	0.65
B12	22.73	5.84	2.11	1.87
B13	0.09	5.84	1.61	0.01
B14	0.00	1.46	—	2.56
B15	9.09	3.65	2.64	0.35
B17	18.18	17.52	1.05	0.05
B18	9.09	0.00	—	6.36*
Bw22	4.55	5.11	0.88	0.41
B27	13.64	16.79	0.78	0.46
B40	4.55	16.06	0.25	3.07
Bw51	4.55	0.73	0.48	0.21

* $P < 0.02$ P value when corrected for antigen specificities tested ($P \times 30$) was > 0.05 and hence not significant.

Table 2. Association of HLA-DR antigens and levels of *Parthenium*-specific IgE antibodies in rhinitis subjects

Subject no.	Sera no. and sex	IgE (PRU/ml)	HLA DR type
1	1186 M	62.5	DR2,DR3
2	1205 M	28.0	DR7,DR-
3	973 M	41.0	DR2,DR4
4	1196 M	162.5	DR3,DR7
5	570 M	105.0	DR7,DR-
6	946 M	140.0	DR2,DR-
7	953 M	50.0	DR3,DR3
8	895 M	117.5	DR1,DR3
9	1072 M	65.0	DR1,DR2
10	1195 M	3.8	DR2,DR7
11	974 M	240.0	DR3,DR5
12	941 F	35.5	DR2,DR3
13	A13 M	9.0	DR1,DR3
14	363 F	25.0	DR3,DR5
15	A21 M	32.5	DR2,DR3
16	616 M	255.0	DR2,DR-
17	A12 M	4.0	DR3,DR7
18	687 M	4.3	DR2,DR3
19	1055 M	43.0	DR3,DR7
20	911 M	117.5	DR3,DR7
21	1207 M	53.2	DR2,DR5
22	A7 M	7.0	DR3,DR7
23	1208 M	33.0	DR2,DR5
24	1075 M	18.0	DR5,DR7
25	948 M	42.5	DR3,DR7
26	1215 M	17.5	DR1,DR2
27	1212 F	22.0	DR2,DR5
28	1024 M	225.0	DR3,DR4
29	Normals (n = 50)	0.35	not included

Parthenium-specific IgE, expressed as PRU/ml was determined by RAST.

and B antigens in rhinitis subjects sensitive to *Parthenium* pollen ($n = 22$) and normal controls ($n = 137$) is shown in Table 1. In comparison with the controls, frequencies of A1, A10, A11, B7, B12, B18 and B51 were found to be higher while Aw19, B5 and B40 were lower in the rhinitis subjects. Among these, only the frequency of HLA-B18 was suggestive of an association with allergic rhinitis ($\chi^2 = 6.33$ at $P = 0.02$). However, this was not significant when corrected for the antigen specificities tested ($P \times 30 > 0.05$).

The phenotypic frequencies of HLA-DR antigen and levels of allergen-specific serum IgE antibodies in *Parthenium*-sensitive rhinitis subjects ($n = 28$) and normal controls ($n = 50$) are shown in Table 2. A comparison of the phenotypic frequencies of HLA-DR antigen in these

Table 3. Comparison of the frequency of HLA-DR antigens in *Parthenium*-sensitive rhinitis patients and control subjects

HLA DR antigen	Subjects (n = 28)		Controls (n = 50)		Relative rate	χ^2
	No.	PF (%)	No.	PF (%)		
DR1	4	14.3	3	6	2.61	0.24
DR2	13	46.4	15	30	2.02	1.41
DR3	17	60.71	6	12	11.33	22.21*
DR4	2	7.1	9	18	0.35	1.37
DR5	3	10.7	6	12	0.84	0.158
DR7	10	35.71	6	12	4.07	3.73
DR-	4	14.28	5	10	0.666	0.527

* $P < 0.0001$

subjects ($n = 28$) and normal controls ($n = 50$) are shown in Table 3. While the frequencies of HLA-DR1, DR2, DR3 and DR7 were higher, those of DR4 and DR5 were lower as compared to the controls. Among these, only the frequency of HLA-DR3 (PF = 60.7%), however, was highly significant ($P < 0.0001$) when compared to controls (PF = 12%). The relative risk of subjects with HLA-DR3 to develop *Parthenium* rhinitis was 11.33 when compared to controls. None of the test subjects had any association with DRw52 or DRw53.

Discussion

Several reports suggest that IgE production against certain allergens such as ragweed, grass or tree pollen, house dust, and mite is genetically regulated [20–24]. It has also been shown that IgE antibody production to Japanese cedar pollen is restricted by the autodominant HLA-linked Is gene [25]. It is now increasingly realized that inheritance of allergy is multifactorial, involving more than one genetic factor [26]. Studies have demonstrated the ability of inbred mice to produce antibodies towards low doses of complex protein antigens which is controlled by at least two distinct sets of genes, namely (1) MHC-linked Ir-genes determining specific antibody responses of both IgE and IgG classes, and (2) an IgE-regulating gene not linked to the MHC complex, where high IgE responsiveness of any antigenic specificity is inherited as a recessive trait [27]. Evidence in humans has also been obtained primarily in the case of responses towards allergens of intermediate molecular weights (10–15 kD), such as *Amb a* III (Ra 3), *Lol p* II and III [7]. Family studies of IgE responses to common inhalant antigens coupled with molecular genetic linkage analysis suggested that atopy is inherited as a dominant trait

[28,29]. In the present investigation, an attempt was made to study the association of HLA and IgE responsiveness to *Parthenium* pollen allergens in subjects with allergic rhinitis. As both HLA class I and II antigens have been shown to play a role in antigen presentation to T lymphocytes and are capable of affecting the early immune responses, it is likely that the manifestation of *Parthenium* rhinitis is MHC regulated.

In view of the strong association between ragweed or rye pollen rhinitis and HLA-DR antigens, and the allergenic crossreactivity between *Parthenium* and ragweed pollen allergens [10–12] attempts were made to study the association of *Parthenium* rhinitis with HLA-DR1, DR2, DR3, DR4, DR5 and DR7 antigens in 28 subjects with elevated levels of IgE and 50 normal subjects. As, for practical reasons, these experiments were performed at a later date, the study group was different from that used earlier for studies with HLA-A and B antigens. A highly significant association between HLA-DR3 antigen and *Parthenium* rhinitis was recorded in 17 out of 28 subjects tested (PF=60.7%) In the present study, the relative risk of having IgE-mediated *Parthenium* rhinitis in unrelated subjects with HLA DR3 was found to be 11.33 times higher than in individuals lacking the antigen (Table 3). These results are similar to those observed for rye and ragweed rhinitis. While the association between HLA-DR3 and allergic rhinitis has been observed mostly for the low molecular weight allergens of ragweed and rye pollen (5–11 kD), a strong association between HLA-DR3 and rye 1 allergen with a molecular weight of 27 kD has also been reported [7]. Although the importance of studying the association of HLA antigens with sensitivity to purified allergens alone has been emphasized, the results of the present investigation can be corroborated with those of Marsh *et al.* [3], since all the *Parthenium*-sensitive subjects recognized the major *Parthenium* pollen allergen with a molecular weight of 31 kD [P. Sriramarao *et al.* unpublished results]. Marsh *et al.* [30] have demonstrated the association between IgE and IgG responsiveness to *Amb a* V allergen and HLA DR2/Dw2 in a large number of patients. Subsequently, it was shown that Dw2 rather than DR2 is associated more strongly with responsiveness to *Amb a* V [4, 31]. Despite a general lack of an antigenic and allergenic relationship between them, the IgE and IgG responses towards the three major rye pollen allergens (*Lol p* I, II and III) were significantly associated with HLA-DR3 and B8 [7]. This may be due to the presence of a similar Ia site on all the three *Lol p* allergens (I, II, and III) that permit concordant recognition of these molecules by an Ia molecule associated with HLA-DR3. Sasazuki *et al.* [25] have described T cells restricted through HLA-DR3 that suppress IgE antibody responses to cedar pollen antigen.

A strong linkage inequality has been reported between HLA-B8 and HLA-DR3 [32] and the influence of such an association can explain the increase in relative risk and also the relatively incomplete nature of most disease association, i.e. subjects having the appropriate HLA antigens but without disease, and vice versa. In the present investigation, however, HLA-A, B and DR typing were done independently and therefore the effect of linkage inequality could not be investigated. HLA-DR3 itself, though significantly associated with IgE response to *Parthenium* pollen allergens, may in conjunction with some other HLA class I and II molecules govern the overall responses to *Parthenium* pollen allergens in patients with allergic rhinitis.

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