HLA-Dw2: A GENETIC MARKER FOR HUMAN IMMUNE RESPONSE TO SHORT RAGWEED POLLEN ALLERGEN Ra5

I. Response Resulting Primarily from Natural Antigenic Exposure*

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We previously emphasized (1, 2) the usefulness of atopic allergy as a model for studying the genetics of human immune response. A particular advantage is that most allergies result from exposure to minute, immunogenically limiting doses of environmental antigens (allergens), usually <1 μg/yr (3). Short ragweed pollen component Ra5 (5,000 mol wt) (4, 5) offers a particularly useful tool for studying specific immune response because of its relatively simple structure and low proportion in the pollen (3). In several studies (2, 6–8), immunoglobulin E (IgE)-mediated skin test response to Ra5 was significantly associated with HLA-B7 and the B7 cross-reacting group (Creg).[1] Further preliminary evidence suggested that the primary association might be with Dw2 (2).

Analysis of the Ra5 preparations used in previous studies by crossed immunoelectrophoresis (CIE) indicated the presence of very low (but detectable) levels of impurities that might interfere with genetic analysis. Hence, further purification of Ra5 to ≥99.9% purity was performed for the present studies, and highly sensitive assays for IgE and IgG antibodies (Ab) to Ra5 were developed.

After studying responses to ultra-pure Ra5 in 447 Caucasians that were naturally exposed to ragweed pollen, we now find that Dw2 is almost a perfect marker for IgE and IgG responses to Ra5. The immune response association with HLA-B7 is secondary to Dw2 and presumably arises from the well-documented linkage disequilibrium between the alleles coding for these two specificities. Analysis of HLA-DR typing data in a portion of the study subjects suggests a weaker association between response to Ra5 with DR2 than with Dw2.

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[1] *Abbreviations used in this paper:* AB, antibody; BSA, bovine serum albumin; BBS, borate-buffered saline, pH 8.3; CIE, crossed immunoelectrophoresis; Creg, cross-reacting group (of HLA antigens); DNV, double-normalized values; Dw2+(−), positive (negative) for HLA specificity Dw2; HLA, human leukocyte antigen; HSA, human serum albumin; Ra5+(−), positive (negative) response to Ra5; RAST, radioallergosorbent test; RIA, radioimmunoassay.

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Materials and Methods

Study Groups. Two groups of unrelated subjects of Euro-Caucasian origin were studied. The first consisted of 86 individuals (50 male, 36 female; mean age 38 ± 10 yr), who were participating in various immunologic and clinical studies of ragweed allergy in the Division of Clinical Immunology (9–13).[2] Serum samples were available on 85 of these 86 individuals. Where patients were to be scheduled for immunotherapy with ragweed antigens (14), sera were collected before therapy and stored at −70°C or −20°C. (It should, however, be noted that 27% of the patients had received immunotherapy with ragweed from their private physician sometime in the past, but not in the 2 yr before serum samples were drawn.) All subjects had been carefully selected because of their severe hay fever toward ragweed pollen, which was well documented by clinical history and symptom diaries. All patients were also strongly positive to short ragweed pollen extract by skin test, histamine release, and radioallergosorbent test (RAST) assays (10).

A second group comprising 361 individuals (271 male, 90 female; mean age 39 ± 10 yr) were employees of the Westinghouse Electric Corporation, Hunt Valley, Baltimore County, MD. These subjects were part of a large genetic-epidemiologic study of human immune responsiveness to allergens, described in detail elsewhere (15). This population was comprised of 119 randomly selected subjects, together with a further 242 subjects selected on the basis of age and residence criteria and of reported allergy toward pollens and/or animals. 11% of 101 ragweed-positive subjects (see Skin Test Procedures) reported having received ragweed immunotherapy in the past, and 4% were currently receiving therapy.

In the Westinghouse population, all study subjects were HLA typed. In the study of clinic patients, we focused on sampling all available Ra5-positive (Ra5+) subjects (from a group of about 300 people) along with appropriate Ra5-negative (Ra5−) (although highly ragweed-sensitive) controls, matched by age, sex, and ongoing therapy with ragweed.

Antigens. Dialyzed extract of short ragweed (*Ambrosia elatior*) pollen was prepared as described elsewhere (15). The preliminary evaluation of skin test response to Ra5 was performed using one or more of three different samples of Ra5 prepared by us or kindly supplied by Dr. Lawrence Goodfriend, McGill University, Montreal, Canada. Although these preparations were homogeneous according to conventional physicochemical and immunochemical analyses (4, 6–8, 16), more discriminating analysis by CIE against concentrated IgG immunoglobulins from rabbits hyperimmunized with crude ragweed extract revealed the presence of one or two minor impurities in all preparations (cf. analysis of preparation Ra5-1 in ref. 17). In view of the extremely high sensitivity of human allergic response to trace (but potentially highly allergenic) contaminants, further purification of Ra5 was deemed necessary.

For the present study, we produced an ultra-pure Ra5 preparation of "isoallergen Ra5A," for which the estimated purity was ≥99.9% (18).[3] In brief, short ragweed fraction C (19), kindly supplied by Dr. Daniel Mullally, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, was further fractionated by ultrafiltration, using a series of Amicon hollow-fiber devices (Amicon Corp., Scientific Sys. Div., Lexington, MA), followed by column chromatography on CM-Sephadex (twice) and Biogel P10 (twice). Contaminating proteins were rigorously monitored and progressively eliminated at each stage of chromatographic fractionation. The final material was homogeneous by gel filtration, agarose gel electrophoresis at pH 8.6, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and CIE, and gave a single amino acid sequence for 20 N-terminal residues (18) corresponding to the published major Ra5 sequence (5).

A comparison by CIE of the final Ra5A isolate vs. a crude 16-min extract of ragweed pollen demonstrated the ultra-purity of the Ra5A preparation (Fig. 1). Although we could detect ~20 different cathodic antigens in the crude extract using a potent anti-ragweed IgG pool raised primarily toward basic ragweed antigens, the Ra5A antigen gave only a single immunoprecip-

[2] Marsh, D. G., E. Ehrlich-Kautzky, and P. S. Norman. 1981. A kinetic study of human immune response toward injection of ragweed allergen and allergoid. Manuscript in preparation.

[3] M. Roebber, D. G. Klapper, and D. G. Marsh. Two isoallergens of short ragweed component Ra5. Manuscript submitted for publication.

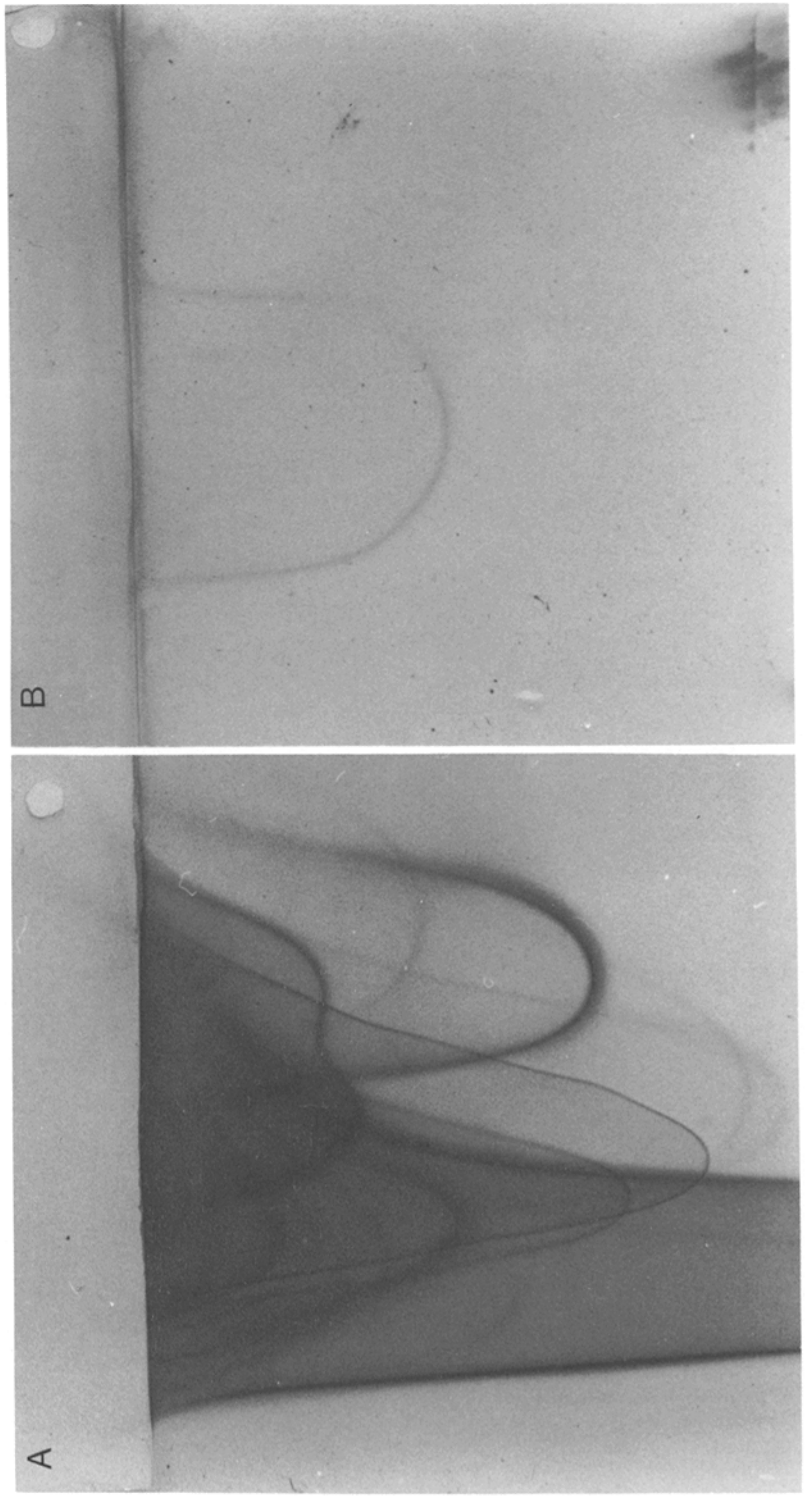

FIG. 1. CIE analyses demonstrating the purity of allergen Ra5A. The left-hand CIE pattern shows cathodically migrating antigens (at pH 8.6) in a dialyzed 16-min extract of short ragweed pollen (100 μg) against a five-times concentrated IgG Ab pool (25 μl/cm^2) from 11 rabbits hyperimmunized with the same extract. The right-hand CIE shows Ra5A (0.5 μg) against the same rabbit antiragweed pool (30 μl/cm^2).

itate.[4] The same result was obtained by Dr. Henning Lowenstein (personal communication) in an independent CIE analysis using a different concentrated rabbit IgG pool raised toward a 24-h extract of ragweed pollen.

Radioimmunoassay of IgG and IgE Ab. Radioimmunoassays (RIA) of IgG and IgE Ab to Ra5 were performed on pretreatment sera, using methodology described in detail elsewhere.[2] In brief, Ra5A was radiolabeled by the chloramine T procedure, using 2 mCi of ^{125}I per 40 μg of Ra5. After separation from unbound iodine by gel filtration and dialysis, the resultant solution was adjusted to 10 μg Ra5/ml in borate-buffered physiological saline (BSS) at pH 8.3, containing 5% bovine serum albumin (BSA) as a stabilizer, millipore-filtered (Millipore Corp., Bedford, MA) to remove micro-precipitates, and stored at −70°C until used.

The double-antibody procedures used to measure IgG and IgE Ab levels consisted of incubating 100 μl of ^{125}I-Ra5 (9 ng/ml in 5% BSA) with 100 μl human serum (1/10 to 1/50 dilution in BBS for the IgG assay or 1/2 dilution for the IgE assay) for 4 h at 23°C. For the RIA of IgG Ab, goat anti-human IgG (γ-chain specific)[5] was added in slight antibody excess, and the mixture was allowed to incubate for 18–20 h at 4°C. The resultant immunoprecipitates were washed and counted.[2] For the IgE Ab RIA, 100 μl of a mixture of myeloma serum PS (1/200) plus a slight excess of goat anti-IgE (ε-chain-specific)[5] was added at the second incubation step (21). The inclusion of the myeloma serum (kindly supplied by Dr. K. Ishizaka, The Johns Hopkins University School of Medicine) was necessary to ensure an adequate amount of precipitate for subsequent washing. Further procedures were performed as in the IgG RIA. Serum IgG and IgE Ab levels were computed by reference to a titration curve, using standard serum of known high IgG Ab content (13) after subtracting values for blank sera (containing no IgG or IgE Ab to Ra5).

All analyses were performed in triplicate in at least two assays. Further experiments were performed where the coefficient of variation was >10% for the means of the first two assays. Sometimes as many as four or five assays were necessary to finalize anti-Ra5 Ab levels in diluted sera for the range of 0.3–1.0 ng/ml.

Total Serum IgE. Total serum IgE levels were analyzed by the direct binding assay of Schellenberg and Adkinson (22, 23). This technique uses anti-IgE-Sepharose beads as an immunosorbent for serum IgE. Bound IgE is subsequently detected using ^{125}I-anti-IgE.

Skin Test Procedures. Intradermal skin test titration, using 0.05 ml of Ra5 at concentrations ranging from 10^{-6} to 1 μg/ml, was performed as previously described (6). The sensitivity of a given individual was defined as the Ra5 concentration eliciting an 8- to 10-mm wheal 15–20 min after antigen injection. Puncture skin testing was performed according to Santilli et al. (24), with modifications (15), using dialyzed short ragweed pollen extract (300 μg/ml), Ra5A (10 μg/ml and 300 μg/ml), and phosphate-saline-human serum albumin diluent control. The 300 μg/ml concentration of Ra5 was used only when reaction at 10 μg/ml was <3-mm wheal Diam. Comparison of the two procedures ussng one-half-$\log_{10}$ increments in antigen concentration showed that puncture testing was ~1 million-fold less sensitive than intradermal testing with respect to the relative antigen concentrations needed to induce end point wheals.

HLA Typing. HLA-A, B, and C typing was performed by standard microcytotoxicity technique using 90 well-defined HLA typing sera obtained from the NIH serum bank and private sources. In the case of the Westinghouse study, a constant panel of HLA typing sera was maintained, with the exception of eight replacements that were well-standardized against the original sera. A somewhat greater number of replacements was permitted for typing the local patients, depending on the availability of particular typing sera over a 3-yr period.

HLA-D typing was performed using well-defined homozygous typing cells obtained from our Amish studies and other sources. HLA specificities Dw1, 2, 3, 4, and 7/11 were each defined by two typing cells, and Dw5 and Dw6 were each defined by one typing cell. The cross-reacting

[4] This figure shows cathodically migrating antigens at pH 8.6. There are also ~40 anodically migrating antigens in short ragweed pollen extract (20). Independent tests showed complete absence of other anodically and cathodically migrating antigens using up to 25 μg of Ra5A.

[5] Antisera used as the second antibody in the RIA were prepared by immunizing goats with the Fc fragments of human IgG or IgE. The anti-IgG serum was adsorbed on an IgG light-chain Sepharose column, where necessary, to remove cross-reacting antibody. The IgG fraction of anti-IgE serum was precipitated with ammonium sulfate and was extensively adsorbed on a human IgG-Sepharose column several times to remove all traces of anti-IgG activity.

specificities Dw7 and 11 (Dw7/11) were grouped together for analytical purposes. With the exception of some of the Westinghouse study group, all individuals were D typed at least twice. The typing results were analyzed by a double-normalization procedure using a computer program reported at the 7th International Workshop (25). By this method, one may calculate the double-normalized values (DNV) for each stimulator-responder combination. In general, cells carrying the same allele as the typing cell gave a DNV below 35%, whereas those not sharing the allele gave a stimulation value of >60%. Ambiguous typing responses were usually resolved by repeated D typing.

HLA-DR typing was performed according to our modification (26) of the method of vanRood et al. (27), using 70 sera of well-defined specificity with which we were able to identify specificities DR1 to DR8, DR10, MB(MT)1, MB2, MB3, and MT2. DR typing was performed on 37 clinic patients and 42 ragweed-sensitive Westinghouse subjects after most of the D typing had been completed. The main objective was to examine the concordance between DR and D typing data in relation to their association with response to Ra5.

Data Analysis. Patients' responses to Ra5A were evaluated primarily according to their serum IgE or IgG Ab levels. Using diluted sera, we were unable to distinguish the cpm values corresponding to Ab levels of 0.3 ng/ml as being significantly different from background cpm using the blank sera. Because 1/10 and 1/2 serum dilutions were the highest concentrations used in the IgG and IgE assays, respectively, we could compute Ab levels in undiluted serum of >3 ng/ml in the case of IgG and >0.6 ng/ml in the case of IgE. Persons having lower than these detectable limits were considered to be negative for all nonparametric statistical analyses and were characterized as having levels of 3 ng/ml and 0.6 ng/ml of IgG and IgE Ab in the case of parametric tests.

Allergic responses to Ra5A were also evaluated by skin testing the local clinic patients, initially using the puncture technique and, subsequently, the intradermal method in about one-third of the patients. Distinct wheal reactions (<3-mm Diam larger than control) on puncture testing with Ra5 at 10 μg/ml or 300 μg/ml were considered positive. Positive reactions seen only at the higher concentration were confirmed by intradermal testing and found to correspond to a sensitivity end point of 10^{-2} to 10^{-3} μg/ml. (At Ra5 concentrations higher than this level, even using ultra-pure Ra5A, reactions could be toward highly allergenic impurities at levels of 0.001 to 0.0001%.)

In both the local and Westinghouse subjects, proportions of the different HLA specificities and the sex distribution Ra5+ vs. Ra5− groups (evaluated by IgE and IgG Ab determinations and skin testing) were compared by Fisher's exact test (two-tailed). Relative risks were usually computed according to the formula $\frac{ad}{bc}$, where a is the number of Ra5+ subjects with the phenotype, b is the number of Ra5+ without the phenotype, c is the number of Ra5− subjects with the phenotype, and d is the number of Ra5− without the phenotype. In cases where b is 0, the formula of Haldane (28), $\frac{2a+1}{2b+1} \times \frac{2d+1}{2c+1}$, was used. A t test (two-tailed) was used to analyze difference in age or log[total IgE] levels between the Ra5+ and Ra5− groups.

A more comphrehensive evaluation of the influence of variables—such as HLA type, log[total IgE], age, sex, and previous immunotherapy on log[IgE Ab] or log[IgG Ab] level—was carried out by a series of stepwise multiple regression analyses using the Biomedical Data Programs package on a DEC-10 (Digital Equipment Corp., Maynard, MA) computer. For each individual, HLA types were categorized as 2, where the specificity was definitely present; 1, where the typing was equivocal (for certain D typings; see later); and 0, where the specificity was absent. Reported immunotherapy (allergen injections, including ragweed) by the patient's clinician was categorized as 2, where injections had been given during the past 5 yr, 1 for injections given >5 yr ago, and 0 for no immunotherapy.

Results

Relationships between IgE Ab, IgG Ab, and Skin Test Response to Ra5. Of 85 local clinic patients for whom serum samples were available, 29 (34%) were found to have detectable IgE Ab, and 34 (40%) detectable IgG Ab to Ra5. Among 361 Westinghouse

study subjects, 101 (28%) were positive to crude ragweed extract (300 μg/ml) by puncture skin test, and, of these, 9 (8.9%) had detectable IgE Ab and 12 (11.9%) had detectable IgG Ab to Ra5. An additional two Westinghouse subjects, who were not positive to ragweed by puncture test, were found to have IgG Ab but no IgE Ab to Ra5. Among the 119 randomly sampled Westinghouse subjects, the prevalence rates for the Ra5 IgE Ab+ and Ra5 IgG Ab+ phenotypes were 1.7% and 3.4%, respectively. The difference in the proportion of Ra5+ persons among the clinic patients and the ragweed-allergic Westinghouse subjects reflects the different selection criteria used (see Materials and Methods).

Each of the frequency distributions of log[IgE Ab] and log[IgG Ab] levels among Ra5 responders for the clinic and the Westinghouse groups was not significantly different from a normal distribution. There were no significant differences in the proportions of males and females who were Ra5+ (categorized by either IgE or IgG Ab response) in either the clinic patients or the Westinghouse study group. The mean ages of Ra5+ vs. Ra5− subgroups were also not significantly different. Among ragweed-positive individuals (either clinic patients or Westinghouse subjects), the mean log[total IgE] levels between Ra5+ vs. Ra5− subjects (by IgE or IgG Ab) were not significantly different. There was, however, a significant difference in the mean log [total IgE] for Ra5+ vs. Ra5− among the total group of Westinghouse study subjects, reflecting the very large proportion of nonallergic subjects with low total serum IgE levels in the Ra5− group.

Only the clinic patients were extensively skin tested with Ra5. Among 18 subjects tested by the intradermal titration procedure, there was a weak correlation ($r = 0.53$, $P < 0.05$) between skin test sensitivity and log[IgE Ab]. All of the skin test-positive clinic patients had detectable IgE Ab to Ra5, except one individual (aged 63 yr) who was weakly skin test positive (intradermal sensitivity at 10^{-2} μg Ra5/ml; 3-mm wheal on puncture testing at 300 μg Ra5/ml). A low level of IgG Ab (4 ng/ml) was found in this patient's serum.

Fig. 2 shows that there is a good correlation ($r = 0.84$, $P < 0.001$) between log[IgG Ab] and log[IgE Ab] response to Ra5 in 34 local clinic and 13 Westinghouse subjects in whom IgG Ab responses to Ra5 were detectable. None of these subjects were

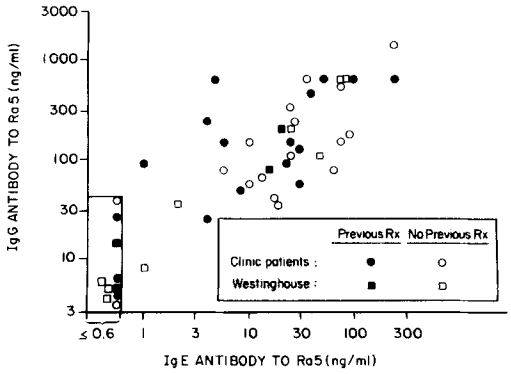

FIG. 2. Relationship between IgG Ab and IgE Ab levels to Ra5 in 47 subjects exhibiting positive IgG Ab responses. Subjects having IgG Ab but no detectable IgE Ab are contained within the box drawn in the bottom left of the graph. One Westinghouse subject, currently on ragweed therapy, was excluded (see text). $r = 0.842$; $P < 0.001$.

currently receiving immunotherapy with ragweed antigens; one Westinghouse Ra5+ subject, who was receiving high dosage ragweed immunotherapy and had an extremely high level of IgG Ab to Ra5, was omitted from this analysis. Only 37 of the 47 IgG Ab+ subjects were IgE Ab+. The correlation between log[IgG Ab] and log[IgE Ab] was less strong ($r = 0.657$, $P < 0.001$) in these 37 study subjects. Among IgE Ab+ subjects, the geometric mean levels of IgG Ab and IgE Ab were 150 ng/ml and 21 ng/ml, respectively; among IgG Ab+ subjects, the corresponding geometric means were 80 ng/ml and 9.8 ng/ml, respectively. The correlation between log[IgE Ab] and log[IgG Ab] among Westinghouse subjects was greater than for the clinic patients ($r = 0.964$ vs. 0.756), probably because more of the clinic patients had previously received immunotherapy with ragweed antigens. The correlation between log[IgE Ab] and log[IgG Ab] was greater for 28 previously untreated vs. 19 previously treated subjects ($r = 0.903$ vs. 0.775).

Analysis of HLA Associations by Nonparametric Statistics. The proportions of all the different HLA types among Ra5+ vs Ra5− individuals were compared using nonparametric statistics (Fisher's exact test, two-tailed). The only significant associations observed in both groups of subjects were with HLA-B7 and Dw2 (Table I). As indicated in the footnote to Table I, HLA-Aw19 was significantly positively associated, and HLA-Dw7/11 significantly negatively associated, with response to Ra5 in the clinic patients. Considering the number of HLA specificities studied, these associations are not very strong. Furthermore, they were not confirmed by analysis of the Westinghouse group. In fact, there was a nonsignificant positive association between Dw7/11 and response to Ra5 in the Westinghouse subjects (see also multiple regression analyses where the association was significant in the Westinghouse subjects).

The striking findings were the particularly strong associations between Dw2 and both IgE Ab and IgG Ab responses to Ra5 in the two study groups. This was especially noteworthy in the case of IgE Ab, where all nine of the Westinghouse Ra5+ subjects and 27/29 of the Ra5+ clinic patients had Dw2. (For all ragweed-positive subjects, $P < 0.0001$, relative risk is 65.) The skin test-positive clinic patient who had no detectable IgE Ab was also found to have Dw2. We retyped the two Ra5+, Dw2− clinic patients twice, and the absence of Dw2 was confirmed.[6]

In view of a preliminary finding (30) showing that the HLA-A3, B7, Dw2 phenotype is significantly associated with the general expression of allergy in the Westinghouse group, we were interested to ascertain whether the associations between various combinations of A3, B7, and Dw2 might be stronger than with Dw2 itself. This was found not to be the case. As shown in Fig. 3, the relative risks of having IgE Ab to Ra5 are much greater among individuals with Dw2 than for any of the other phenotypic combinations of A3, B7, and Dw2 in clinic patients, Westinghouse ragweed-positive subjects, and the total Westinghouse group.[7] Similar findings were noted in analyzing the IgG Ab data (not shown). These results clearly show that the primary HLA association with response to Ra5 is with Dw2 and not with combinations of HLA antigens.

[6] In a preliminary report of these data (29), we had identified three Ra5+ clinic patients as being Dw2−. One of these three individuals, who had left the area, was recently located and was retyped twice using three Dw2 homozygous typing cells in each experiment. Both experiments showed unequivocally that he was Dw2+ by all three typing cells.

[7] The Haldane (28) formula was used to compute data for Dw2 in the Westinghouse groups where no Ra5+ subject was Dw2−.

TABLE I

Associations between HLA and Immune Responses to Ra5 in Clinic and Westinghouse Study Patients *

Study group	Number of subjects		Percent with B7		*P* values	Percent with Dw2		*P* values
	Ra5+	Ra5−	Ra5+	Ra5−		Ra5+	Ra5−	
1. Local clinic patients; IgE Ab‡	29	56	52	29	0.056	93	18	<0.0001
2. Local clinic patients; IgG Ab	34	51	50	27	0.041	85	16	<0.0001
3. Local clinic patients; skin test‡	30	56	53	27	0.019	93	16	<0.0001
4. Ragweed-positive Westinghouse; IgE Ab	9	92 (83)§	56	25	0.11	100	24	<0.0001
5. Ragweed-positive Westinghouse; IgG Ab	12	89 (80)§	50	25	0.087	83	24	0.0001
6. All Westinghouse subjects; IgE Ab	9	352 (326)§	56	22	0.033	100	21	<0.0001
7. All Westinghouse subjects; IgG Ab	14	347 (321)§	50	22	0.022	79	21	<0.0001
Combined groups 1 and 4; IgE Ab	38	148 (139)§	53	26	0.004	95‖	22	<0.0001
Combined groups 2 and 5; IgG Ab	46	140 (131)§	50	26	0.004	85	21	<0.0001

* Significant positive associations ($0.05 > P > 0.01$) between immune responsiveness to Ra5 (detected by IgE Ab, IgG Ab and skin test) were seen in the local clinic patients for HLA-Aw19 (combined A29, Aw30, Aw31, Aw32, and Aw33), and significant negative associations ($0.05 > P \geq 0.01$) were seen with HLA-Dw7/11 (Dw7 and/or Dw11). These associations were not confirmed in the Westinghouse subjects (see text).

‡ Pretreatment serum was not available on one Ra5− patient. Also, one weakly skin test-positive subject had no detectable IgE Ab; hence the numbers in the Ra5+ and Ra5− categories are not identical.

§ Westinghouse subjects having equivocal typings for HLA-Dw2 (9 Ra5− subjects in the ragweed-positive and 26 in the total Westinghouse study group) were included in the B7 but excluded from the Dw2 analyses (see text). The reduced numbers given in parentheses reflect the subjects for whom definitively positive or negative typings for Dw2 were obtained.

‖ The D/DR typings on the two Dw2− individuals were: Dw1/DR1, Dw?/DR?, MB(MT)1; Dw?/DR4, Dw?/DR8, MB?, MT2.

Multiple Regression Analyses. To obtain more discriminating analyses of the data sets, relating HLA etc. to the magnitude of the IgE Ab or IgG Ab response to Ra5, we carried out a series of stepwise multiple regression analyses. The different HLA types, age, sex, previous ragweed immunotherapy, and log[total serum IgE] were the independent variables and log[IgE Ab] or log[IgG Ab], the dependent variables. The following groups were analyzed: clinic patients, ragweed-positive Westinghouse subjects, and all Westinghouse subjects. HLA-Dw2 showed by far the strongest associations with log[IgE Ab] and log [IgG Ab] to Ra5 in both study groups, with *P* values of 10^{-6} to 10^{-14}. In addition, log[IgG Ab] was significantly associated with previous immunotherapy. Among one or the other ragweed-positive groups, less striking (but significant) associations were observed with A2, A3, B5, B14, Bw35, Cw3, and Dw7/11, of which A2 and B14 were negatively associated with response to Ra5. In the total Westinghouse group, log[total IgE] was also significantly associated with response to Ra5 because of the large proportion of nonallergics in the Ra5− group.

We wished to compare, critically, the relative influence of each of the variables significantly associated with response to Ra5 in at least one of the analyses of IgE Ab

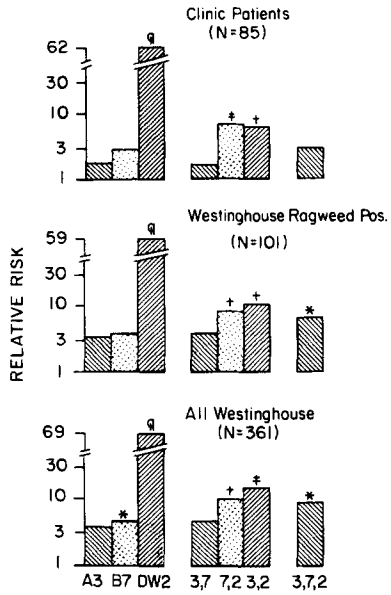

FIG. 3. Comparison of relative risks for IgE Ab response to Ra5 in persons having phenotypes HLA-A3, B7, Dw2, or various combinations thereof. Significant associations are indicated as follows: * $0.05 \geq P > 0.01$; † $0.01 \geq P > 0.001$; ‡ $0.001 \geq P > 0.0001$; ¶ $P < 0.0001$.

TABLE II

Multiple Regression Analyses Examining Relationships between Log[IgE Ab] and Log[IgG Ab] Responses to Ra5 and the Independent Variables Found to Be Significant in Previous Stepwise Multiple Regressions

Independent variable	Clinic patients (N = 85)*				Westinghouse ragweed (N = 97)‡			
	log[IgE Ab]		log[IgG Ab]		log[IgE Ab]		log[IgG Ab]	
	b§	P‖	b	P‖	b	P	b	P
A2	−0.138	0.047	−0.125		0.074		0.063	
A3	−0.029		−0.038		0.223	0.020	0.204	0.028
B5	0.246	0.001	0.271	3.3×10^{-4}	−0.022		−0.020	
B14	−0.233	0.001	−0.171	0.017	0.126		0.109	
B27	−0.029		−0.030		0.215	0.018	0.215	0.015
Bw35	0.201	0.005	0.106		0.082		0.042	
Cw3	0.197	0.007	0.107		−0.063		−0.058	
Dw2	0.691	1.8×10^{-14}	0.698	1.6×10^{-14}	0.430	7.9×10^{-6}	0.446	2.1×10^{-6}
Dw7/11	−0.075		−0.081		0.276	0.003	0.295	0.001
Previous Rx	0.051		0.155	0.034	0.114		0.192	0.033

* Pretreatment serum was not available on one clinic patient.
‡ *D*-locus typing data were not available on four subjects.
§ Standardized regression coefficient.
‖ Two-tailed tests. Only values of $P \leq 0.05$ are cited.

or IgG Ab responses in ragweed-positive individuals. Therefore, in a series of multiple regression analyses, we investigated the association between each of these variables and log[IgE Ab] or log[IgG Ab] in clinic patients and in Westinghouse employees. The results (Table II) show that only Dw2 is significantly associated with response to

Ra5 in all of the categories studied (P values of 10^{-5} to 10^{-14}). In addition, the influence of previous immunotherapy with ragweed is evident for the analyses of IgG Ab response both for the clinic and Westinghouse subjects. For all other independent variables, significant associations ($P < 0.05$) were found either in the clinic or the Westinghouse group, but not simultaneously in both groups. No significant associations were found between response to Ra5 and the presence of HLA-B7 in any of the multiple regression analyses. This is because of the association ("arising from" linkage disequilibrium) between HLA-B7 and Dw2, which precludes finding an association between response to Ra5 and B7 in the presence of the overwhelming association between Ra5 response and Dw2.

DR-D Relationships. Finally, we investigated whether DR typing data could clarify the HLA associations further. We DR typed 79 subjects (37 clinic and 42 Westinghouse), of whom 13 clinic and 7 Westinghouse subjects had detectable IgE Ab to Ra5. The people who were DR typed were not random samples of the two groups; they were chosen to include as many Ra5+ subjects as possible, especially the two Ra5+, Dw2− subjects. Analysis of the data by Fisher's exact test showed that the primary DR association with IgE Ab response to Ra5 was with DR2 ($P < 0.0001$; relative risk, 19). The association of IgE Ab response to Ra5 with Dw2 in these same 79 subjects was more striking ($P < 0.0001$; relative risk, 29). DR5 was negatively associated with IgE Ab to Ra5 ($P < 0.02$); no Ra5+ but five Ra5− subjects had DR5. Similar results were obtained in analyzing IgG Ab data.

The IgE Ab data showed complete concordance between typings for Dw2 and DR2 for all 20 Ra5+ subjects studied. We found that the two exceptional Ra5+, Dw2− clinic patients were also DR2−. Among the 59 Ra5− subjects, DR2 was present in 32% and Dw2 in 24% of the cases. Four Ra5− clinic patients and one Westinghouse subject were DR2+, Dw2−; none was DR2−, Dw2+. There were no unusual MB/MT-DR relationships for subjects having either the Ra5+, DR2−, Dw2− or the Ra5−, DR2+, Dw2− phenotypes.

Discussion

In two different groups of Caucasian subjects, we demonstrated significant, extremely strong associations between human immune response to short ragweed allergen Ra5 and possession of HLA-Dw2. In the case of IgE Ab response, the association is particularly striking; overall, 95% of 38 Ra5+ vs. 22% of 139 Ra5− ragweed-allergic subjects possessed Dw2 ($P < 0.0001$; relative risk, 65). These conclusions were confirmed by the use of multiple regression analyses, where Dw2 was found to be strongly correlated with the magnitude of the log[IgE Ab] and log[IgG Ab] levels. Associations were significant with P values of $\sim 10^{-14}$ in the clinic patients and 10^{-5} to 10^{-6} in the Westinghouse ragweed-allergic subjects (Table II).

The Dw2-Ra5 association is by far the strongest HLA association that has been observed with a defined immune response in humans. The association is of similar magnitude to that found between B27 and ankylosing spondylitis (31), a disease where there is no clear-cut association with a specific immune response. Allergy to Ra5 requires production of anti-Ra5 IgE Ab; furthermore, there is a concordant production of specific IgG Ab (in sevenfold higher concentration) in IgE Ab+ subjects. Some additional subjects make low levels of IgG Ab but no detectable IgE Ab to Ra5. As noted with response to other inhaled allergens (21, 32, 33), there is a good

correlation between log[IgG Ab] and log[IgE Ab] to Ra5 in responder subjects not currently receiving allergen immunotherapy (Fig. 1). The somewhat weaker association between Dw2 and IgG Ab response in the nonparametric (but not the multiple regression) analyses might result from the influence of previous immunotherapy with ragweed antigens in many of the study subjects. The significant influence of previous immunotherapy on IgG Ab response is clearly evident from the multiple regression analyses (Table II).

The crucial elements in the success of these studies were (*a*) the use of an ultra-pure (≥99.9% pure) sample of a defined polypeptide antigen of relatively simple structure (45 amino acids) (5), and (*b*) the development of highly sensitive RIA procedures to measure serum IgE Ab and IgG Ab responses. It should be emphasized that, in the highly polymorphic human population, studies using partially purified (ca. 95% pure) or highly complex protein antigens might not yield informative data (7).

In previous studies (6, 7), we observed a significant association between allergic skin test response to Ra5 and HLA-B7. It now appears that the primary association is with Dw2 and that the B7 association results from the linkage disequilibrium between B7 and Dw2. In view of several reports (26, 31) suggesting stronger associations between various autoimmune diseases and DR (as opposed to D) specificities, it is interesting to find that Dw2, rather than DR2, is more strongly associated with response to Ra5. Among subjects who were DR typed, no discordance was observed in 20 Ra5+ subjects between DR2 and Dw2; 18 of 20 had the DR2, Dw2 phenotype, and the remaining two individuals were negative for both specificities. On the other hand, among 59 Ra5− individuals, 5 of 18 (28%) of the subset who typed DR2+ were Dw2−. Other data (34) suggest that, in Caucasian populations, ~24% of DR2+ subjects do not have an associated Dw2 specificity.

The finding of a large number of Ra5−, Dw2+ subjects raised the obvious question of whether there might be other genetic factors (as well as the obvious need for environmental exposure) determining response to Ra5. Also, could such factors be circumvented by artificial immunization with much higher dosages of Ra5 than encountered as a result of natural inhalation of pollen grains? The widespread use of immunotherapy with ragweed antigens for the treatment of ragweed allergy provides a ready-made experiment to test the latter hypothesis. The results of such an investigation, together with mechanistic interpretations of the Ra5-Dw2 association, are presented in the succeeding paper.

Summary

Ultra-pure short ragweed pollen allergen Ra5 (5,000 mol wt) was used to investigate the relationship between human leukocyte antigen (HLA) type and IgE and IgG antibody (Ab) responses to Ra5 in two groups of Caucasian subjects, totaling 447 people. Using highly sensitive radioimmunoassay procedures to measure serum IgE and IgG Ab, qualitative responses to Ra5 in both groups were found to be strongly associated with HLA-Dw2 ($P < 0.0001$). For example, 95% of 38 people with IgE Ab vs. 22% of 139 ragweed-allergic persons having no detectable IgE Ab to Ra5 were Dw2+. Quantitative log [IgE Ab] and log[IgG Ab] responses to Ra5 were highly correlated with Dw2 ($P = 10^{-5}$ to 10^{-14}) in four separate multiple regression analyses, examining the relationship between HLA type (and other variables) and Ab levels in the two study groups. Further studies showed that the primary association of Ra5

response was with Dw2 rather than DR2 and that various combinations of A3, B7, and Dw2 were less strongly associated than Dw2 alone.

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