

RAGWEED ALLERGEN Ra5: A NEW TOOL IN UNDERSTANDING THE GENETICS AND IMMUNOCHEMISTRY OF IMMUNE RESPONSE IN MAN*†

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Throughout his distinguished career, one of the continuing interests of Dr Dan Campbell was in the immunochemistry of allergens from a wide variety of different natural sources. He was also closely involved in several studies of artificial allergens produced through the interaction of haptens (usually from drugs) and proteins. Through his appreciating the biological significance of such studies, he helped to keep alive the training of young people in allergen immunochemistry. His central consideration was that chemical studies of all facets of immediate hypersensitivity research are essential for a more complete understanding of the subject and its relationship to our general appreciation of the mechanisms of immune response. It was my great pleasure to share in part of this work.

D.G.M.

Abstract—Immunoglobulin E-mediated skin sensitivity to a minor short ragweed pollen allergen, Ra5, was found to be highly significantly associated with the possession of histocompatibility antigen HL-A7 and with antigens of the HL-A7 Cross-reacting group in a study of 139 ragweed-allergic Caucasians. This finding was supported by a smaller second study in a different location. This research lays the groundwork for future immunochemical investigation of the genetic basis of immune response in man.

INTRODUCTION

The historical notion that atopic allergy is a unique type of immunological disease mediated by a unique subset of antigens responsible for the production of 'reagin' (cf. Augustin, 1959; Berrens, 1971) has tended to set allergy research apart from the mainstream of immunology. These old ideas should be reevaluated following discoveries that: (i) reaginic activity is associated predominantly with a specific class of immunoglobulin, IgE (Ishizaka and Ishizaka, 1968), and (ii) recent advances in immunogenetics which strongly suggest that IgE antibody response in animals (including man) is under a similar genetic control as that of IgG, at both the T and B cell levels (Levine and Vaz, 1970; Marsh *et al.*, 1973a; Kindt and Todd, 1969; Gally and Edelman, 1970). Furthermore, it is now established that the dosages of most naturally inhaled allergens (e.g. pollen components) fall in the range of nanograms/year (Marsh *et al.*, 1973b; Marsh, 1975a). Such low dosages would be expected to be immunogenically limiting, which is a

requirement for observing the effect of specific immune response genes determining responsiveness in some individuals but not others (Levine and Vaz, 1970; Benacerraf and McDevitt, 1972). An evaluation of these considerations and the long-established familial aggregation of allergy suggest that research utilizing atopic allergens will have a unique role to contribute to our understanding of the overall genetics of immune responsiveness in man (Marsh, 1974, 1975b; Black and Marsh, 1975).

An important step in this direction came with the isolation of a highly purified protein allergen, Ra5, from the pollen of short ragweed (Goodfriend and Lapkoff, 1972; Lapkoff and Goodfriend, 1974). This component possesses the simplest chemical structure of any well-characterized allergen (mol. wt = 5000 daltons). Only about 20 per cent of ragweed-allergic subjects were found to be sensitive to Ra5, and serum IgG antibody response accompanied that of IgE (Marsh *et al.*, 1973a, 1973b, 1973c). Most importantly, responsiveness to Ra5 was found to be closely associated with the possession of a major histocompatibility antigen of the HL-A7 Cross-reacting group (Creg) comprising HL-A7, W-27, W-22, and W-10. This was the first demonstration of a significant association between a specific immune response and a specific group of HL-A antigens in man. The early work was carried out on 105 subjects (of which 97 were Caucasians of European extraction), and it was essential

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that they be confirmed in further studies, including the use of different Caucasian population samples. We have, therefore, extended our original studies of the ragweed-allergic population living in the Baltimore area to include 30 Ra5 allergic subjects; in addition, we have made a preliminary study of a different population sample located in Belleville, Illinois. In both of these studies, we find a marked elevation of HL-A7 itself as well as HL-A7 Creg in people who are highly allergic to Ra5, the elevations being highly significant in the larger Baltimore group.

MATERIALS AND METHODS

Allergens

Antigen E (AgE), isolated from the pollen of short ragweed (*Ambrosia elatior*), was initially provided by Dr T P King, Rockefeller University, New York. The antigen was isolated in his laboratory by standard published procedures (King *et al.*, 1964, King, 1972) and was shown to be of high purity by physical, chemical and immunological analysis (reported in Marsh *et al.*, 1973c). Later experiments were performed using an AgE preparation from the NIH Research Reagents Division, which was further purified by Sephadex G75 gel filtration. This material was also found to be homogeneous by extensive immunological analyses against several anti-AgE and anti-whole ragweed sera. By electrophoretic analysis, the NIH preparation consisted mainly of isoallergenic subcomponents IV-A, B and C with traces of IV-D, whereas Dr King's preparation was mainly IV-C*. Solutions of the two samples adjusted to the same optical density of 280 nm had the same AgE concentrations by radial immunodiffusion analysis (Marsh, 1975a).

Allergen Ra5 was isolated from short ragweed pollen according to the method of Lapkoff and Goodfriend (1974). All preparations used were homogeneous by previously reported physico-chemical and immunological criteria (Marsh *et al.*, 1973c, Lapkoff and Goodfriend, 1974) and gave a single symmetrical peak on isoelectric focusing in a glycerol gradient (Fig. 1).

Patient selection

The present studies were limited to Caucasians, because of the marked genetic differences between different races, including the distribution frequencies of different HL-A antigens. All subjects reported marked allergic symptoms to ragweed pollen, indicating that they had previously received adequate exposure to the pollen and had a genetic predilection toward allergy.

Baltimore, Maryland study

One hundred thirty-nine unrelated, highly allergic subjects (79 M, 60 F, aged 12–57) were obtained from ongoing clinical and immunologic studies of ragweed hay fever and asthma at The Johns Hopkins and Good Samaritan Hospitals. Of these, 97 had been analyzed in a previous study (Marsh *et al.*, 1973c). The patients were already known to be highly sensitive by skin test and/or leukocyte histamine release to ragweed pollen extract, and most had been

previously selected for their high sensitivity to AgE. About one-third had previously been subjected to immunotherapy with ragweed extracts. All patients possessed 2+ intradermal sensitivity at 10^{-3} μ g/ml or lower (see below) to one or both of these allergens.

Belleville, Illinois study†

Ninety-eight unrelated Caucasian subjects reporting ragweed hay fever symptoms and reacting positively by prick test to crude ragweed extract were submitted for study. Of 80 people possessing significant sensitivity to AgE and/or Ra5 (2+ or greater prick reaction) 72 (90 per cent) were significantly sensitive to AgE and 30 (37 per cent) to Ra5. Blood samples were drawn for HL-A typing on 37 of the 80 highly sensitive individuals, with a substantial bias toward including a high proportion of Ra5-sensitive subjects.

Most of the Baltimore group had lived in the area for the majority of their lives, but the Belleville population, consisting entirely of Air Force personnel, were born and had travelled in many different parts of the United States and abroad. The mean annual ragweed pollen exposures in Baltimore and Belleville are believed to be similar‡. The incidence of different species of ragweed (possessing somewhat different AgE and Ra5 components) varies widely throughout the United States (Wagner, 1959). In the Baltimore area, by far the most predominant species is *Ambrosia elatior* (short ragweed), from which the allergens used for the study were extracted, but in other areas, such as Belleville, other species are also abundant‡. From the foregoing discussion, it is clear that the antigenic stimulation was much more uniform in the Baltimore population.

Analysis of IgE-mediated sensitivity

Two skin test methods for analyzing IgE-mediated sensitivity were used. In the Baltimore study, patients were investigated by quantitative intradermal skin test titration (0.05 ml injection), using solutions of AgE and Ra5, as previously described by Marsh *et al.* (1973c). A patient's skin sensitivity is defined as the allergen concentration (μ g/ml) which elicits a reaction graded as '2+' (0.8–1.0 cm wheal with 2.0–4.0 cm erythema, 15–20 minutes following injection). Using this data, patient's skin sensitivity ratios, Ra5/AgE, were calculated. The importance of using sensitivity ratios, rather than absolute sensitivity to Ra5, to compensate for individual differences in ragweed pollen exposure and general genetic predilection toward allergy is discussed elsewhere (Marsh *et al.*, 1973c, Marsh, 1975b). Patients' intradermal skin sensitivity ratios were found to differ by no more than ten-fold from ratios determined more quantitatively by leukocyte histamine release in Dr L. M. Lichtenstein's laboratory at Johns Hopkins University School

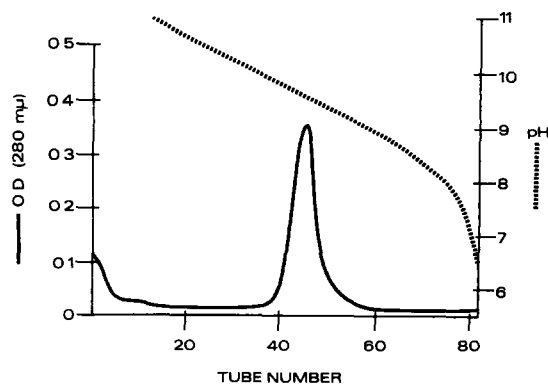


Fig. 1 Isoelectric focusing of antigen Ra5, using a glycerol gradient. Conditions: sample, 10 mg, ampholine range, pH 9–11, running time, 4 days, vol./tube, 1.1 ml. (This experiment was kindly performed by Marianne Roebber.)

* The isoallergenic variants of AgE have indistinguishable immunological properties but vary slightly in isoelectric point (King *et al.*, 1964, Johnson and Marsh, 1965).

† A detailed account of other clinical and immunological aspects of this study will be presented elsewhere (Santilli *et al.*, 1975 in preparation).

‡ The pollen counts in the two geographical areas are similar (unpublished observations). However, in Belleville the proportion of *Ambrosia trifida* (giant ragweed) is much higher than in Baltimore.

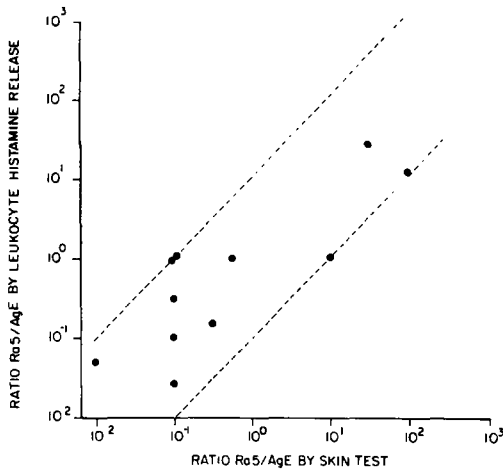


Fig. 2 Comparison of allergic sensitivity ratios, Ra5/AgE, determined by quantitative intradermal skin testing and leukocyte histamine release in 11 Ra5-sensitive subjects. All subjects tested have points falling within the dashed lines, representing 10-fold differences between the two ratios. Linear regression analysis $r = 0.82$, $p < 0.01$.

of Medicine (Fig. 2). This shows that intradermal skin titration is quite a precise technique.

In the Illinois study, all patients were prick tested, using solutions of antigens E and Ra5 at 30 $\mu\text{g}/\text{ml}$ in Tris-albumin buffer (Santilli *et al.*, 1975). Prick test reactions were graded 1+ to 5+ according to wheal diameter. Intradermal skin sensitivity was found to relate almost linearly with the prick test grading (Santilli *et al.*, 1975), which allowed Ra5/AgE sensitivity ratios to be estimated fairly precisely simply by subtracting the prick test grading for Ra5 from that for AgE, provided both reactions were positive.

HL-A Typing

In the Baltimore study, leukocytes were harvested from heparinized blood samples taken less than 3 hr previously. In the Illinois study, sterile samples were rushed by jet from Illinois to Baltimore and typing was performed the next day. Typings were performed by standard cytotoxic microtechnique (Amos *et al.*, 1969) using 58–90 sera of well-defined specificities obtained from the NIH and private sources. Only analyses of 22 major specificities are reported, although sub-specificities of several HL-A types were recognized by the sera used (see legend to Table 1).

RESULTS

Figure 3 shows the distribution of Ra5/AgE skin sensitivity ratios in the Baltimore ragweed-sensitive population. Highly Ra5-sensitive and Ra5-insensitive subjects fall into two distinct groups, A and B respectively, which represent 22 per cent and 60 per cent of the population studied. For the purpose of analyzing HL-A antigen frequencies, people having intermediate ratios of greater than 3×10^2 , but less than 10^5 (on a weight basis), were omitted. The 300-fold difference in sensitivity ratio between Ra5-sensitive Group A and Ra5-insensitive Group B is sufficient to avoid possible misclassification of patients due to experimental error or possible reactions to trace allergenic impurities in Ra5. Such impurities, although undetected by rigorous physical and chemical analysis

(Fig. 1; Lapkoff and Goodfriend, 1974; Mole *et al.*, 1974b) may be detectable by the sensitive skin test assay in some people. Beside analyzing for HL-A differences between Groups A and B, analysis was also made between matched Groups A and B'. Group B' was obtained by individually matching (without knowledge of HL-A type) 30 subjects from Group B with those of Group A, based on age, sex, sensitivity to AgE, degree of previous immunotherapy and occurrence of ragweed asthma.

Among the Belleville population, 12 subjects reacting positively to Ra5 possessed Ra5/AgE sensitivity ratios estimated to be 10 or less and five had ratios between 10 and 100. The remaining 20 subjects had ratios of greater than 100 and were classified as being Ra5-insensitive. The criterion for Ra5 insensitivity is, however, not as rigorous as the Baltimore study, since the insensitive group includes 11 individuals who were only moderately sensitive to AgE (2+ prick reaction). Although these subjects were negative to Ra5 at the allergen concentration used, a few may react positively at a 10 or 100-fold higher concentration (and, thus, should more appropriately be classified as 'intermediately Ra5-sensitive').

The frequencies of HL-A serotypes in the Ra5-sensitive and Ra5-insensitive populations from the two studies are presented in Table 1. Comparison of the frequencies of each HL-A specificity, in Group A vs B or B' and Group α vs β , showed that the only significant differences* were in the frequencies of HL-A7 (Table 2). The incidence of HL-A7 in Ra5-sensitive Group A in the Baltimore study was 2.8 times higher than the unmatched Group B and 5 times higher than the matched Group B' of Ra5-insensitive subjects; in both cases, the differences were highly significant. In the Belleville study, the frequency of HL-A7 was higher in Group α than Group β , but this was not

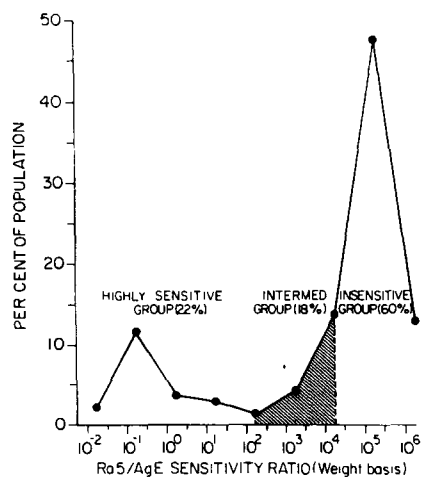


Fig. 3 Distribution of Ra5/AgE sensitivity ratios in the Baltimore ragweed-sensitive population ($N = 139$). In determining the population frequencies, individuals possessing ratios of 10^{-2} have been pooled with those having ratios of 3×10^{-2} , 10^{-1} with 3×10^{-1} , etc. Intermediately Ra5-sensitive individuals possess weight ratios greater than 3×10^2 but less than 10^5 . Weight ratios may be converted to molar ratios by multiplying by 0.13. The distribution shown may not represent that of a truly randomly selected ragweed-allergic population, since many of the patients were originally collected on the basis of their high leukocyte responsiveness to AgE.

* With $P \leq 0.05$, using chi-squared analysis with Yates' correction for small sample sizes.

Table 1 Comparison of absolute frequencies of HL-A antigens in ragweed-allergic populations from Baltimore, Maryland and Belleville, Illinois. There are significant differences in certain HL-A7 and HL-A7 Creg distributions (Table II)

HL-A Serotype	BALTIMORE			BELLEVILLE		
	Ra5 sensitive		Ra5 insensitive	Ra5 sensitive		Ra5 insensitive
	Group A (N = 30)	Group B (N = 84)		Group A (N = 17)	Group B (N = 20)	
1st (Ls) Locus						
1	8	24	6	6	4	4
3	11	23	8	4	4	4
11	2	7	3	2	1	1
10*	2	15	6	1	~	~
2	14	40	17	9	9	9
W-28	1	3	0	1	0	0
9*	3	15	7	2	5	5
W-19*	5	6	2	3	7	7
2nd (Four) Locus						
5	4	9	1	1	3	3
W-5	2	2	1	1	6	6
W-18	1	7	2	~	1	1
W-15	1	1	1	1	0	0
12**	2	30	11	4	2	2
13	1	6	4	0	1	1
W-10**	4	8	4	4	3	3
W-27**	1	6	1	3	2	2
7	15	15	3	4	3	3
W-22	1	3	1	1	0	0
W-16	1	4	1	1	0	0
W-17	3	4	0	2	0	0
8	6	23	8	3	2	2
W-14	2	9	2	1	2	2

Brackets indicate Cross-reacting groups (Cregs) of HL-A antigens (see Marsh *et al.*, 1973b for further details). Among 7 American Blacks, of mixed Caucasian and Negro descent, who were studied in addition to the Caucasians reported in this table, 1 was found to be Ra5 sensitive and 6 insensitive. The sensitive subject was typed as HL-A7, two of the insensitive subjects were positive for HL-A7 and one for W-22

* Includes antigens serologically recognized as W-25 and W-26 (variants of HL-A10)

~ Includes antigens serologically recognized as W-23 and W-24 (variants of HL-A9)

† Includes antigens serologically recognized as W-29 through W-32 (variants of W-19)

** We could distinguish, serologically, two variants of each of these specificities, some of which are discussed in Marsh *et al.* (1973c)

significant. When the frequency of the HL-A7 Cross-reacting group (Creg) of related HL-A antigens in each group was examined, it was highly significantly elevated for Group A vs Group B or B'. In the Belleville study, the incidence of HL-A7 Creg was 1.6 times higher in Group α than Group β ; however, the sample size was too small for meaningful statistical analysis. In further analysis, only people in each group who did not have the specificity defined as HL-A7 were compared. Here, the combined frequencies of the other 7 Creg specificities (W-10, W-22 and W-27) were about twice as high in the Ra5-sensitive groups relative to the insensitive groups (Table 2). Due to the smaller populations, these differences were not significant at the 0.05 level.

DISCUSSION

Marked IgE-mediated sensitivity to the low mol wt. short ragweed pollen allergen, Ra5, is present in about 22 per cent of the Baltimore ragweed-allergic population. For people who are subject to different

exposure, in terms of total pollen load and different species of ragweed, the incidence of Ra5 sensitivity may well be substantially higher, as evidenced from the Belleville study where the incidence is 37 per cent. Reactivity to Ra5 tends to occur in an 'all or none' fashion (Fig. 3) suggesting the possibility that responsiveness to Ra5 may be under a fairly simple genetic control. Statistical analysis revealed that IgE-mediated sensitivity to Ra5 is closely associated with the possession of HL-A7 and, most probably, with other histocompatibility antigens comprising the HL-A7 Creg. In the larger study conducted in Baltimore, HL-A7 itself is highly significantly elevated in Ra5-sensitive relative to Ra5-insensitive people ($P = 0.001$), and when the combined frequencies of HL-A7 Creg antigens are considered, the elevation is also highly significant ($P = 0.003$).

In the Baltimore study, 70 per cent of Ra5-sensitive and 37 per cent of all Ra5-insensitive subjects possess one or more HL-A7 Creg antigens. The corresponding figures from the smaller preliminary Belleville study are 65 per cent and 40 per cent respectively. Although, in the latter case, the difference is not statistically significant, the overall percentages agree closely with the Baltimore results. The preliminary Belleville findings are particularly encouraging in view of the marked heterogeneity of previous ragweed exposure in that population (see Patient Selection). Taken together, these observations provide strong evidence that an immune response (Ir) gene (or group of closely related Ir genes), controlling recognition of Ra5, is closely associated with the possession of an HL-A7 Creg antigen, in keeping with a previous study in a smaller population sample (Marsh *et al.*, 1973c). By analogy with previous animal studies, the hypothesized 'Ir-Ra5 gene(s)' may control immune recognition at the cellular level of a principal carrier determinant on the Ra5 molecule. The marked association between immune responsiveness to Ra5 and the possession of HL-A7 Creg suggests the existence of significant 'linkage disequilibrium' between closely linked genes coding for HL-A7 Creg antigens and postulated 'Ir-Ra5' gene product(s) (Bodmer, 1973; Marsh, 1975b).

Not all Ra5-sensitive individuals possess HL-A7 or another antigen of the HL-A7 Creg. These individuals, comprising 30% of the Baltimore Group A and 35% of Belleville Group α , may be explained as follows: (i) in some individuals, 'major Ir-Ra5 gene(s)' are not associated with HL-A7 Creg, perhaps due to previous genetic recombination; (ii) 'minor Ir-Ra5 gene(s)' associated with other histocompatibility antigens, perhaps controlling recognition of subsidiary Ra5 determinants, may be involved; (iii) the recognition of Ra5 molecules from different ragweed species may be under different genetic control (cf mouse studies of Hill and Sercatz (1974), using lysozymes from different species of birds as antigens).

Some highly allergic, but Ra5-insensitive, individuals possess HL-A7 Creg antigens. This may arise possibly by recombination between 7 Creg and postulated Ir loci, or when an individual does not have other genes essential for the development of specific IgE antibody response to Ra5 (Marsh *et al.*, 1973b; Bias, 1973; Marsh, 1975b).

The finding of such a close relationship between HL-A7 and HL-A7 Creg and specific immune response to Ra5 in ragweed-allergic subjects probably

Table II Statistical analysis of HL-A7 and HL-A7 Creg frequencies in Ra5-sensitive and Ra5-insensitive subjects in the Baltimore and Belleville studies (see Table I). All p values were calculated by chi-squared analysis using Yates' correction

HL-A	Percentage HL-A Frequencies					
	Ra5-sensitive			Ra5-insensitive		
	A	α	B	B'	B	P values
7	50	24	18	10	15	0.001 0.002 N.S.
7 Creg*	70	65	37	27	40	0.003 0.002 N.S.
W-10, W-22 & W-27†	40	54	23	19	29	N.S. N.S. N.S.

N.S. = Not significant at the $p = 0.05$ level

* Includes HL-A7, W-27, W-22 and W-10 (Marsh *et al.*, 1973c). The percentages reflect the proportions of people who have 7 Creg genes on one or both chromosomes.

† The percentages cited represent the frequencies of people possessing at least one of these antigens in the subpopulations of each group who do not possess HL-A7 itself (e.g., 7/15 for Group A)

results from the exquisitely limiting conditions of natural immunization, due to the simple molecular structure and low dose of Ra5 (mean inhaled dose in the Baltimore area <60 ng/year; Marsh, 1975a). Under such circumstances, it is possible to demonstrate highly significant associations which are not evident for higher dosage exposure to highly complex antigens such as Antigen E (Marsh and Bias, 1974).

Allergen Ra5 is only one of a number of different highly purified pollen allergens we have been investigating for associations between HL-A and specific IgE response in allergic populations. We have found significant associations for responses to allergens of intermediate complexity, but these are more pronounced for individuals who possess genetically limiting low total serum IgE levels (Marsh *et al.*, 1974; Marsh and Bias, 1974; Marsh, 1975b). Despite these findings, we have so far been unable to establish genetic linkage between familial HL-A haplotypes and cellular or humoral immune responsiveness to any one of the allergens studied in extensive research with allergic and non-allergic families (Bias *et al.*, 1973; Marsh *et al.*, 1974; Black *et al.*, 1974, 1975). Our failure to demonstrate linkage and conflicting studies purporting to show linkage have been critically reviewed by Bias and Marsh (1975), Black and Marsh (1975) and Marsh (1975b).

Due to the simple chemical structure of Ra5, it has recently been possible to determine the complete amino acid sequence, within a few months, using only 1.5 μ mole of material (Mole *et al.*, 1974, 1975). The availability of such sequence data, particularly on simple molecules like Ra5, should greatly facilitate future investigation of the chemistry of immune recognition, both at the T- and B-cell levels. This may eventually lead to the possibility of mapping the genetics of immune response in man. Toward this end, it seems already apparent that immunochemical studies of allergens will play a vital role.

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