

TWO ISOALLERGENS OF SHORT RAGWEED COMPONENT Ra5¹

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Two isoallergenic forms of short ragweed (*Ambrosia elatior*) allergen Ra5 were isolated in ultrapure form by a combination of molecular sieving (through Amicon hollow-fiber cartridges), cation-exchange chromatography, and gel filtration. The two forms, Ra5A and Ra5B, exhibited charge differences by CM-Sephadex chromatography and agarose electrophoresis at pH 8.5. The A form was identical to conventional Ra5 by its physical and chemical properties and was more cationic than the previously undescribed B form. Ra5A and Ra5B had identical m.w. of 5000 by SDS-PAGE. Their amino-terminal sequences of 30 residues showed no differences from each other or from the previously published sequence of the major form of Ra5. The two forms were antigenically indistinguishable when using hyperimmunized animal antisera to Ra5 and crude ragweed extract. Although the IgG and IgE ab of most Ra5-responder patients could not discriminate between the two Ra5 forms, significant small differences in binding to Ra5A and Ra5B were consistently observed in 10 to 15% of the patients studied. Further analysis of the Ra5A preparation suggested a purity of at least 99.5% and probably $\geq 99.9\%$.

Most isolated allergens have been shown to exist in multiple molecular forms that exhibit slight physicochemical differences but have indistinguishable allergenic properties. Such allergenic variants have been termed isoallergens (1, 2). Among the short ragweed pollen allergens, AgE² and AgK are each known to exist in two or more electrophoretic variants (3–5), and Ra3 has two forms that differ by size rather than by charge (6). The existence of several distinct forms of an allergen with (apparently) identical allergenicity suggests that certain chemically variable regions of an allergen molecule may play little or no role as allergenic determinants in man. Therefore, chemical and immunologic analyses of isoallergens are of interest in relating chemical structure to allergenic activity in man.

Short ragweed pollen allergen Ra5, a small (5000-dalton) basic protein (7) with a known primary sequence of 45 amino acids (8), has been used as a model allergen in a number of genetic studies (9, 10). The latest work in our laboratory

revealed a remarkably strong association between immune response to Ra5 and possession of HLA²-Dw2; 36/38 Ra5-allergic subjects have Dw2, and all Dw2+ Ra5-nonallergic subjects make IgG antibody (ab) after ragweed therapy (11, 12). The simple structure of Ra5 offers an approach to the study of the chemical prerequisites for the observed HLA association.

In the present paper, we describe the preparation and properties of two isoallergens of Ra5, Ra5A (the previously isolated form) and Ra5B (a new isoallergen) differing from Ra5A by charge at pH 8.5. Both allergens were isolated in a sufficiently pure form to allow quantitative comparison of their antigenic and allergenic relatedness in terms of their reactivity with human IgG and IgE ab. Ra5A was shown to be ultrapure (est. $\geq 99.9\%$ pure) by several criteria including crossed immunoelectrophoresis (CIE).

MATERIALS AND METHODS

Preparation of ragweed pollen RW (16-min) extracts. Ten percent (w/v) extracts of two lots of nondefatted *Ambrosia elatior* (short ragweed) pollen (Greer 18-50-72 and 18-P5-69) were prepared by stirring 1 g of pollen with 10 ml H₂O for 16 min at 23°C. Each extract was passed through an 0.45- μ m Millex filter unit and was used immediately for agarose electrophoretic experiments (see below). Dialyzed RW (16-min) extracts used for immunizing rabbits were prepared by extracting petroleum-ether defatted pollen with borate-buffered saline (BBS) for 16-min (13), dialyzing extensively against 0.002 M NH₄HCO₃ and finally distilled water, and lyophilizing.

Preparation of Ra5. An ammonium sulfate precipitate of short ragweed fraction C (Fr. C) (3) was obtained with the kind assistance of Dr. Daniel Mullally, NIAID. Three-kilogram dry pollen equivalents of Fr. C were dissolved in distilled water (6 liters) and were centrifuged. The solution was found to contain 144 mg Ra5/kg dry pollen equivalent by radial immunodiffusion (RID) analysis (see below). The solution was ultrafiltered sequentially through two Amicon hollow-fiber cartridges with nominal m.w. exclusion of 10,000 and 2000. The material that passed through both cartridges (Fr. III) was concentrated to 225 ml using an Amicon UM2 membrane.

Fr. III (225 ml, containing 315 mg of Ra5) was applied to a CM-Sephadex C-50 column (5 x 28 cm) and was eluted in stepwise fashion using increasing concentrations of sodium phosphate buffer (0.02, 0.04, and 0.1 M) at pH 7.0. The eluates of this (and subsequent) chromatography experiments were monitored by OD_{280 nm}, immunodiffusion (ID) against specific anti-Ra5A serum and agarose electrophoresis at pH 8.6 (see below). Ra5-containing fractions were concentrated to 50 ml by UM2 filtration and were recycled through a fresh column of CM-Sephadex (2.5 x 28 cm) equilibrated with 0.02 M phosphate buffer. The Ra5A- and Ra5B-containing CM-Sephadex fractions (15 ml each) were separately passed through a Biogel P10 (200 to 400 mesh) column (2.5 x 88 cm). The major OD_{280 nm} peak in each case containing all the Ra5 was concentrated to 15 ml and was recycled through the same column.

Antisera. Anti-RW (16-min) serum was raised by hyperimmunizing 11 rabbits with dialyzed RW (16-min) extract (20 mg in 1 ml saline), emulsified with Freund's adjuvant (1 ml) containing 10 mg of *M. butyricum*. Injections were administered subcutaneously in multiple sites. Depending on the antibody response, this immunization schedule was repeated two to five times at 3-wk intervals. Subsequent booster injections were given every 3 wk using 5 mg RW (16-min) in 1 ml saline emulsified with 1 ml Freund's adjuvant containing 1 mg *M. butyricum*. The rabbits were bled 1 wk after every booster injection. Goat monospecific antiserum to Ra5A was prepared as described previously (13). In the case of both the anti-RW (16-min) and anti-Ra5 sera, bleeds with the highest titers were pooled, precipitated with ammonium sulfate (25 g/100 ml), and the resulting IgG cuts were concentrated fivefold compared to the original serum volumes.

Agarose electrophoresis. Analytic agarose electrophoresis at pH 8.5 or

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² Abbreviations used in this paper: ab, antibody; AgE, antigen E; AgK, antigen K; BBS, borate-buffered saline; CIE, crossed immunoelectrophoresis; CLIE, crossed-line immunoelectrophoresis; Fr. C, fraction C of short ragweed pollen; Fr. III, fraction III of short ragweed pollen; HLA, human leukocyte antigen; %, percent inhibition; ID, immunodiffusion; RID, radial immunodiffusion; RIA, radioimmunoassay; RW (16-min), 16-min extract of ragweed pollen.

8.6 was performed at 10°C essentially as described by Laurell (14) by using 1% agarose (Seakem, Type ME, FMC Marine Colloids Div., Rockland, ME) in Tris-barbital buffer (Type CIV, Sigma Chemical Co., St. Louis, MO) on plastic sheets (Gel Bond, FMC). After completion of the electrophoresis runs (60 min at 10 V/cm), the protein components were fixed and stained for 1 hr³ in a solution of 0.9% Coomassie Blue (R-250, Bio-Rad Laboratories, Richmond, CA) in 25% methanol, 10% trichloroacetic acid, 3% sulfosalicylic acid, and 61% H₂O. The slides were destained in a solution of 7.5% acetic acid, 10% methanol in H₂O.

In other experiments, Ra5 was specifically identified by overlaying the agarose plates with the concentrated IgG fraction of goat anti-Ra5A (0.07 ml/cm²) immediately after electrophoresis. After allowing the serum to react for 6 hr at room temperature in an incubation chamber, the plates were washed overnight in saline, dried, and stained.

ID and immunoelectrophoresis (IEP). ID and quantitative RID were carried out as described elsewhere (15, 16). For IEP samples were run for 60 min at 10 V/cm in 1% agarose gels in Tris-barbital buffer at pH 8.6, and antiserum was added to troughs cut in the gels, which were then incubated overnight at 23°C. The gels were washed, dried, and stained with Coomassie Blue. CIE and tandem CIE were performed at 10°C essentially according to Axelsen *et al.* (17). The first-dimension electrophoresis was run at pH 8.5 for 50 to 60 min at 10 V/cm, and the second dimension was run for 14 to 16 hr at 0.5 V/cm.

A "fast" CIE technique was used to quantitate Ra5A and Ra5B in the two RW (16-min) extracts. In this variation of the standard procedure, electrophoresis in the second dimension was performed for 6 hr at 4 V/cm. Solutions of Ra5A standards were run on a separate plate under the same conditions, and a calibration graph of peak height vs concentration of Ra5 was plotted. Concentrations of Ra5A and Ra5B in the RW (16-min) extracts were then determined from the calibration graph.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in slab gels according to the method of Laemmli (18). All chemicals were obtained from Bio-Rad. A linear polyacrylamide gradient from 5 to 20% (prepared with a 100-ml LKB gradient maker) was used as the separating gel. The separating gel dimensions were 1.5 mm (thickness) x 115 mm (length), with a 3% stacking gel of 15-mm length. The average running time was 6 hr at 40 mA and 23°C. The separated proteins were fixed in the gels and were stained overnight by using the solutions described for agarose electrophoresis.

Amino-terminal amino acid sequencing. The Ra5A and Ra5B preparations were also investigated by automated sequencing of the 30 amino-terminal amino acids. The methodology used was as described for a similar analysis of Ra3 by Klapper *et al.* (19), except that high pressure gas chromatography was not included among the analytic techniques used for identification of the phenylthiohydantoin derivatives of the amino-terminal residues.

Double-antibody radioimmunoassay (RIA). The relative binding of Ra5A and Ra5B to human IgG and IgE ab was studied in the following groups of patient sera: group 1 consisted of 25 sera from patients who were skin test-positive to Ra5A and B, but had never received hyposensitization treatment with ragweed; group 2 consisted of eight post-treatment sera from the patients in group 1; group 3 was comprised of 12 post-treatment sera from Ra5 skin test-negative persons who developed IgG ab (but no IgE ab) to Ra5 after treatment with ragweed (12). Serum from a nonallergic, Ra5-negative subject was also included as a control.

Two types of assay were used to assess the relative binding of Ra5A and Ra5B to the antibodies in the sera of these patients—direct-binding assays and inhibition assays. Groups 1–3 were studied in the direct IgG-binding experiments and group 1 in the direct IgE-binding experiments. Five sera from group 1 and two sera from group 2 were used in the inhibition experiments. All analyses were carried out in triplicate in at least two experiments. Assays were repeated when the coefficients of variation within or between experiments were greater than 10%.

For the direct-binding assays (11), ¹²⁵I-labeled Ra5A or Ra5B (100 µl, 9 ng/ml) was allowed to incubate for 4 hr at 23°C with allergic human serum (100 µl), diluted 1/10 to 1/50 for the study of IgG ab and 1/2 to 1/5 for the study of IgE ab. For the IgG assay, 100 µl of a slight excess of goat anti-human IgG (γ-chain-specific) were added. For the IgE assay, 100 µl of a mixture containing IgE myeloma serum PS "carrier" (1/200) and a slight excess of goat anti-IgE (ε-chain-specific) were added. In both types of assay, the immune complexes were allowed to precipitate overnight at 4°C. The precipitates were washed four times with BBS, counted in a gamma counter, and the ab contents were computed by comparison against a standard serum (11).

In a second type of assay, the inhibition of binding of radiolabeled Ra5A to human IgE and IgG antibodies was studied with unlabeled Ra5A or Ra5B used as inhibitors. Radiolabeled Ra5A (100 µl, 9 ng/ml) was preincubated for 1 hr with 100 µl of diluted serum (1/10 to 1/50 dilution in BBS for the IgG assay or 1/2 dilution of the IgE assay. Solutions of unlabeled Ra5A and Ra5B (100 µl, containing 0.06 to 0.8 µg Ra5/ml) were then added and

the mixtures were incubated for 4 hr more at 23°C. Anti-IgE (plus IgE myeloma serum) or anti-IgG was then added as in the direct-binding assay, and the resultant immunoprecipitates were subsequently washed and counted. Percent inhibition (%) was calculated by using the formula:

$$\%I = \frac{(\text{cpm for test serum without inhibitor}) - (\text{cpm for test serum with inhibitor})}{(\text{cpm for test serum without inhibitor}) - (\text{cpm for control serum})} \times 100$$

RESULTS

Detection of two Ra5 components. Figure 1A shows an example of a stained immunoprecipitation pattern obtained when a nondialyzed RW (16-min) extract was fractionated by agarose gel electrophoresis and immunofixed with the concentrated IgG fraction of anti-Ra5. Two distinct bands were obtained; the stronger, more cathodic band had a mobility identical to "conventional" Ra5 (Ra5A). The presence of a less cathodic band (Ra5B) was detected for the first time. This new component was also found in ragweed Fr. C. The fainter staining of the Ra5B band indicated that it was present in lower concentration than Ra5A in both RW (16-min) extract and Fr. C. The antigenic relatedness of the two Ra5 components was investigated by IEP of both RW (16-min) extracts against anti-Ra5A. The precipitin arcs corresponding to Ra5A joined with those of the less cathodic Ra5B in reactions of complete antigenic identity (Fig. 1B).

These experiments demonstrated the presence of two isoelectric variants of Ra5, which were antigenically indistinguishable from one another using goat anti-Ra5A serum. By using the "fast" CIE technique described in *Materials and Methods*, the relative proportion of Ra5A and Ra5B in both RW (16-min) extracts was found to be approximately 4:1.

Preparation of ultrapure Ra5A and Ra5B. Ultrafiltration through hollow-fiber cartridges permitted rapid processing of an amount of Fr. C equivalent to 3 kg dry pollen. Surprisingly, the major part of Ra5 (82%) was located in Fr. III (which passed through the 2000-dalton cartridge) rather than in fractions of higher nominal m.w. Subsequent concentration of Fr. III by Amicon UM2 filtration led to minimal loss of Ra5 (ca. 10%).

Figure 2 shows the CM-Sephadex chromatograph of Fr. III. The less cationic Ra5B was eluted in the long trailing portion of the first peak, and Ra5A was eluted with the next (0.04 M sodium phosphate) buffer step. Recycling of each Ra5-con-

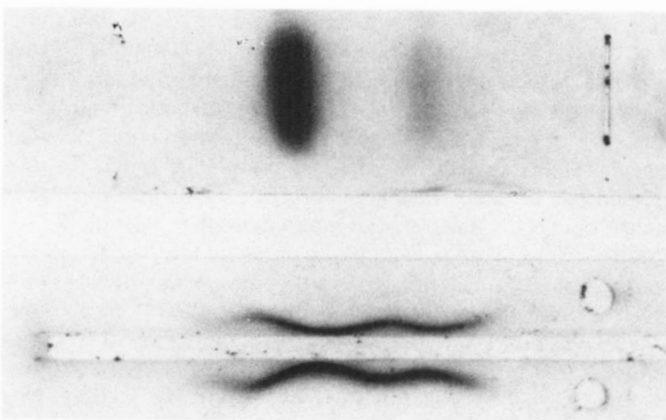


Figure 1. Immunoelectrophoretic analysis of RW (16-min) extracts (10% w/v). A, Agarose electrophoresis at pH 8.6 of extract 18-P5-69 (8) for 1 hr at 10 V/cm, the electrophoretogram was overlaid with specific goat anti-Ra5A serum (IgG cut) before staining (see text). B, IEP of 8 µl of extracts 18-P5-69 (upper well) and 18-50-72 (lower well). Electrophoresis conditions were as described in A. Subsequent development of the precipitin arcs was obtained by applying goat anti-Ra5A serum to the trough.

³ Ra5 washed out of the agarose gels when fixing and staining were carried out for longer than 1 hr.

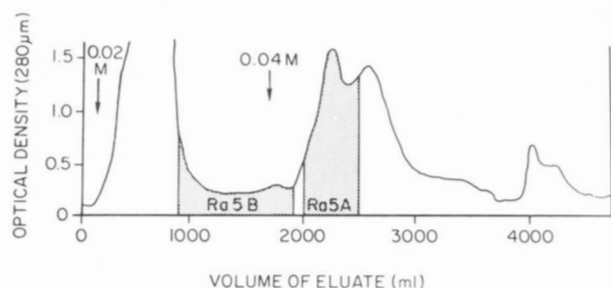


Figure 2. Separation of Ra5A and Ra5B by CM-Sephadex column chromatography. 200 ml of Fr. III were applied to a CM-Sephadex C50 column (5 x 28 cm), equilibrated with 0.02 M phosphate buffer, pH 7.0. A second buffer step (0.04 M phosphate) was used to eluate Ra5A after 1200 ml of the first buffer had been passed through the column. Ra5-containing fractions were pooled as indicated and were recycled through a second CM-Sephadex column (2.5 x 28 cm).

taining fraction through CM-Sephadex using only one buffer concentration (sodium phosphate, 0.02 M) was necessary to ensure purity by charge. The resulting OD_{280 nm} chromatograms showed single, very extended peaks. The center portions, containing the purest Ra5 materials, were selected for further purification.

The partially purified fractions of Ra5A (100 mg) and Ra5B (40 mg) were independently concentrated by UM2 filtration and were fractionated by using Biogel PM10 column chromatography (Figs. 3A and B). Each chromatogram showed a number of small peaks (containing impurities) and a final large Ra5-containing fraction. Only the peak tubes of this fraction, which were free from trace contaminants as judged by ID against anti-RW (16-min) and agarose electrophoresis, were included in the Ra5A or Ra5B pool. Each pool was concentrated and passed through the same column for a second time, yielding single, sharp, homogeneous peaks. Contaminants were not detected in any of the fractions; however, to minimize any remaining trace contamination, the ascending portions of the peaks were not included in the Ra5A and Ra5B pools. The yields of ultrapure Ra5A and Ra5B were 60 and 6 mg, respectively.

Homogeneity and antigenic relatedness of Ra5A and Ra5B.

The purity of the Ra5 isolates was investigated by agarose electrophoresis at pH 8.5 (Fig. 4A). Only one band for each of the Ra5 forms is apparent on the electrophoretogram, showing a high degree of purity by charge. To test the homogeneity by size, SDS-PAGE was also performed with loads of Ra5A and B of 10 μ g and 8 μ g, respectively (Fig. 4B). Because Ra5A was available in large amounts suitable for use in further studies, its homogeneity was further tested by using overloads of 40 μ g and 100 μ g (Fig. 4C). These and other experiments showed that the Ra5 preparations were homogeneous by size at the concentrations used and that the proteins had indistinguishable m.w. (about 5000 daltons by comparison with protein markers).

Antigenic relatedness and purity of the two Ra5 components were studied by CIE against our hyperimmune rabbit serum IgG concentrate, anti-RW (16-min) at 50 μ l/cm². This serum was able to precipitate some 20 basic components of RW (16-min) extract (11). On the other hand, Ra5A and Ra5B (0.5 and 1 μ g) both gave only single precipitin bands (not shown). The tandem CIE technique was employed to show the relatedness and purity of the Ra5 components (Fig. 5). It is clear that the two forms of Ra5 cannot be distinguished antigenically with this serum, and that they contain no detectable antigenic impurities using loads of 0.5 μ g Ra5A and 0.4 μ g Ra5B.

To obtain a further estimate of the antigenic purity of Ra5A,

additional CIE experiments were performed (Fig. 6). The intent was to compare the antigenic composition of the low m.w. Fr. III (from which Ra5 was derived) to a large overload of Ra5A using our potent rabbit IgG pool, anti-RW (16-min). Under optimal conditions, 12 basic components were found in 25 μ g of Fr. III (Fig. 6A). When a large overload of Ra5A (60 μ g) was run under similar experimental conditions, it formed a large diffuse immunoprecipitate peak, only a portion of which is visible (Fig. 6B). There was no evidence of any impurities.

To investigate the possibility of Ra5A contamination in

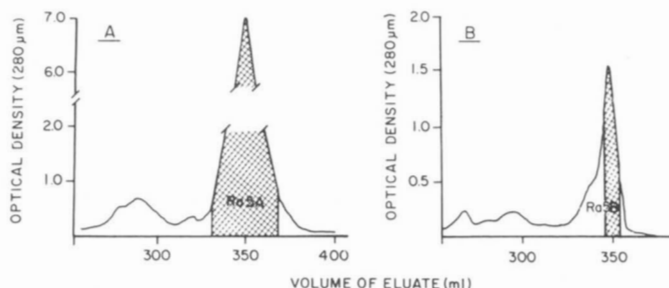


Figure 3. Purification of Ra5A- and Ra5B-containing fractions (from repeated CM-Sephadex chromatography) on a Biogel P10 column (2.5 x 88 cm) equilibrated with 0.05 M Tris buffer, pH 7.6. A, Ra5A. B, Ra5B. Ra5-containing fractions were pooled as shown and were subsequently recycled through the same column.

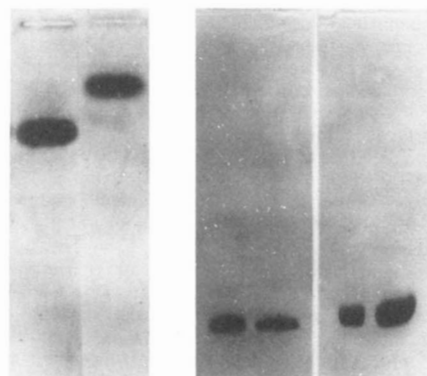


Figure 4. Electrophoretic analysis of Ra5. A, agarose electrophoresis of Ra5A (2.5 μ g) and Ra5B (2.5 μ g) at pH 8.6 for 60 min at 10 V/cm. B, SDS-PAGE analysis of Ra5A (10 μ g) and Ra5B (8 μ g), for 6 hr at 40 mA using a 5 to 20% acrylamide gradient. C, SDS-PAGE analysis of Ra5A with high loads of protein (40 and 100 μ g) to show the homogeneity of this preparation. Electrophoretic conditions were as in B.

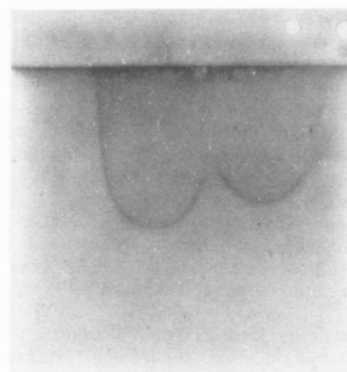


Figure 5. Tandem CIE analysis showing antigenic identity of Ra5A (left) and Ra5B. Ra5A (0.5 μ g) and Ra5B (0.4 μ g) were applied to two wells 0.5 cm apart and run for 1 hr at 10 V/cm in the first dimension (anode to the right). Electrophoresis in the second dimension was for 16 hr at 0.5 V/cm into a gel containing the IgG concentrate of anti-RW (16-min), 50 μ l/cm². The anode is at the top of the figure.

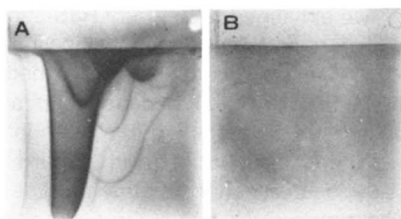


Figure 6. CIE analysis of the purity of Ra5A. Electrophoresis conditions were as described in Figure 5, except that the antiserum was used at 30 μ l/cm². A, 25 μ g of Fr. III (the low m.w. fraction from which Ra5A was prepared) were applied into the antigen well. B, an overload of 60 μ g of Ra5A was applied to the well.

Ra5B and vice versa, both allergens were tested by agarose electrophoresis at pH 8.6 in amounts of 40, 80, 160, and 1600 ng. After electrophoresis (60 min at 10 V/cm), the plates were overlaid with specific goat anti-Ra5A and were developed and stained as described in *Materials and Methods*. At amounts of 40, 80, or 160 ng, both antigens gave narrow bands of immunoprecipitate similar to those seen in Fig. 1A, with the density of staining being proportional to the quantity of Ra5. At 1600 ng loads, Ra5A and B both showed wide bands devoid of staining in the central portion, which is indicative of soluble complex formation in antigen excess. Experiments in which Ra5A (40 to 1600 ng) and Ra5B (40 to 160 ng) were used showed single bands in all cases. However, Ra5B (1600 ng) showed a second component at the position of Ra5A, which stained with a similar intensity to the Ra5A band at 40 ng. By means of this highly sensitive test (estimated to be about 10 times more sensitive than Coomassie Blue staining), we estimated that Ra5A contained less than 1% of Ra5B, whereas Ra5B contained about 3% of Ra5A.

Structural analysis. The primary focus of these preliminary studies was to try to identify amino acid difference(s) between the two forms of Ra5 that could account for the charge difference. Analysis by agarose electrophoresis at a range of pH values suggested that the difference resided in a glutamine or asparagine residue(s) (Klapper *et al.*, unpublished). Another potential difference between Ra5A and Ra5B resides in position 2 from the amino terminus, where Mole *et al.* (8) reported that approximately 70% of Ra5 had a valine and 30% had a leucine. Although this represents an insignificant difference with respect to charge, it was of interest to examine whether these two species of Ra5 had been separated.

Amino-terminal sequence analysis of the first 30 amino acids of Ra5A and Ra5B showed that each preparation had an identical, unique amino-terminal sequence through all 30 steps. These identical sequences were also the same as the major published amino acid sequence of Ra5 with valine at position 2 (8). There was no detectable leucine at position 2 in either preparation as reported previously (8).

Isoantigenicity and isoallergenicity. Previously we showed that, by IEP the two Ra5 forms present in freshly prepared ragweed extracts were antigenically indistinguishable when developed against goat antiserum to Ra5A (Fig. 1B). An analogous result was obtained for the highly purified Ra5A and Ra5B isolates in the tandem CIE experiment using rabbit anti-RW (16-min) (Fig. 5). The aim of the next series of experiments was to investigate quantitatively the allergenic and antigenic relationships between Ra5A and Ra5B with respect to their abilities to bind human IgE and IgG ab.

Figure 7A shows a comparison of RIA results for the direct binding of radiolabeled Ra5A and Ra5B to IgG ab for 45 sera of 37 ragweed-allergic patients. There were no significant differences in the relative binding of Ra5A and Ra5B in sera from untreated patients (group 1; open symbols) or the two

groups of ragweed-treated subjects (closed symbols), as assessed by the slopes of the three regression lines (Table I). More importantly, the correlation coefficients in all three cases were ≥ 0.99 , and the slopes of the regression lines of log(IgG ab to Ra5A) vs log(IgG ab to Ra5B) were not significantly different from 1.00. These results show good overall concordance in the binding of Ra5A and Ra5B to human IgG ab. In three independent experiments, however, we found consistent differences of 13 to 40% in IgG ab binding to Ra5A and Ra5B ($p \leq 0.05$ by *t*-test) for two pretreatment and four post-treatment sera (indicated by arrows in Fig. 7A). One of the two pretreatment sera (E.Ke.) also showed a significant post-treatment difference ($p < 0.02$); the other person (K.S.) was not treated. In the case of two of the remaining post-treatment sera

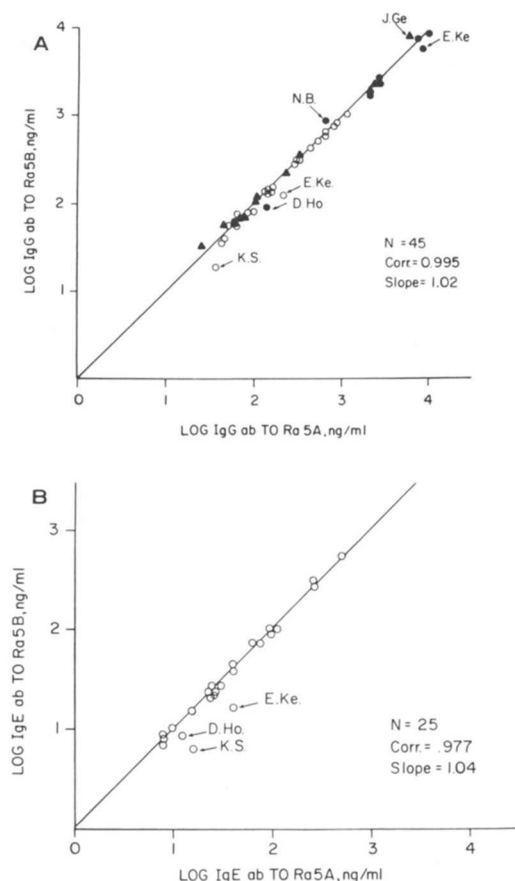


Figure 7. Comparison of levels of human antibodies to Ra5A and Ra5B obtained using direct-binding RIA. A, levels of IgG ab in three groups of sera: group 1 ($\circ$), 25 sera from untreated Ra5-allergic patients; group 2 ($\bullet$), 8 sera from patients in group 1 after ragweed immunotherapy; group 3 ($\blacktriangle$), 12 post-treatment sera from Ra5-nonallergic patients who made IgG ab responses to Ra5. B, levels of IgE ab in the 25 sera of group 1. Persons indicated by arrows in both figures showed consistent significant differences in ab binding to Ra5A vs Ra5B.

TABLE I

Linear regression analysis of the log(Ab) responses to Ra5A and Ra5B				
Group	Antibody Assayed	N	Correlation Coefficient ^a	Slope of Regression Line ^b
1 ^c	IgG	25	0.990	1.07
2 ^d	IgG	8	0.995	1.03
3 ^e	IgG	12	0.997	1.02
1 ^c	IgE	25	0.977	1.04

^a All *p* values are less than 0.01.

^b All slopes were not significantly different from 1.00.

^c Sera from untreated Ra5-allergic patients.

^d Sera from treated Ra5-allergic patients (from a subset of patients in group 1).

^e Sera from treated Ra5-nonallergic patients who made IgG ab (but no IgE ab) responses to Ra5.

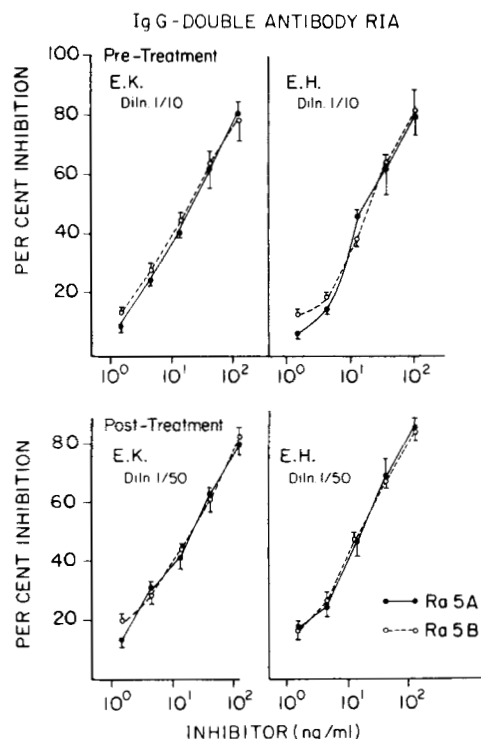


Figure 8. Inhibition RIA comparing the relative abilities of Ra5A and Ra5B to inhibit the binding of ^{125}I -Ra5A to IgG ab in pre- and post-treatment sera of two patients.

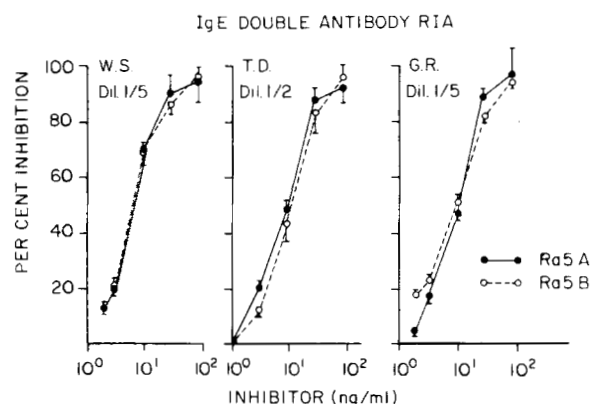


Figure 9. Inhibition RIA comparing the relative abilities of Ra5A and Ra5B to inhibit the binding of ^{125}I -Ra5A to the IgE ab of three untreated persons.

(N.B. and D.Ho.), a significant difference in IgG ab binding was not seen before treatment. Subject J.Ge. could not be studied before treatment.⁴

The 25 allergic sera of group 1 were tested by RIA for the ability of their IgE ab to bind to Ra5A and Ra5B (Fig. 7B). The amounts of ab bound to Ra5A and B were, again, highly correlated ($p = 0.977$), and the slope of the regression line was not significantly different from 1.00. Comparison of the IgE ab to Ra5A and B showed significant differences for E.Ke. ($p < 0.001$), D.Ho. ($p < 0.02$), and K.S. ($p < 0.001$), all three of whom exhibited significant differences in IgG ab binding using pretreatment and/or post-treatment sera. The levels of pretreatment IgE ab of patient N.B. to Ra5A and Ra5B were indistinguishable (cf. IgG ab pretreatment values).

The relative abilities of Ra5A and Ra5B to inhibit binding of ^{125}I -Ra5A to human IgG and IgE ab were also compared with the use of several sera from five persons allergic to Ra5. For

the IgG assay, we examined two sera of Ra5-sensitive patients E.K. and E.H. before and after hyposensitization with ragweed extract (Fig. 8). The sera were used in higher dilution after therapy (1/50 instead of 1/10), because the amount of IgG ab to Ra5 had increased. The binding of IgG ab in both pretreatment sera was identically inhibited with either Ra5A or Ra5B. Furthermore, hyposensitization treatment made no difference to the inhibition patterns. We tested three more treated patients in the same assay and obtained identical results (not shown). Figure 9 shows the inhibition of binding of IgE ab to ^{125}I -Ra5A by cold Ra5A and Ra5B using sera from three untreated patients. As in the case of the IgG assay, inhibition of IgE ab binding by Ra5A and Ra5B was not significantly different. Similar results were obtained with further pretreatment sera from the remaining two patients (not shown).

DISCUSSION

Two electrophoretic variants of allergen Ra5 (A and B) were detected in fresh 16-min extracts of short ragweed pollen by agarose electrophoresis followed by overlaying with a goat anti-Ra5A serum (Fig. 1A). These two forms of Ra5 were antigenically indistinguishable when tested by IEP against the same serum (Fig. 1B). These results led us to isolate both forms of Ra5 in a highly purified state, and to study their biologic and chemical relatedness. One of the forms (Ra5B) had not been described; the other (Ra5A) turned out to have the same physicochemical properties as those described for Ra5 by Lapkoff and Goodfriend (7).

The two variants were antigenically indistinguishable by tandem CIE against our potent rabbit anti-RW (16-min) IgG pool (Fig. 5) and goat anti-Ra5A. In RIA experiments using ^{125}I -Ra5A or ^{125}I -Ra5B, there was close similarity between the two forms with respect to their direct binding of most human IgG and IgE ab ($r \geq 0.99$; Table I, Fig. 7). A few subjects, however, appeared to be capable of recognizing unique determinants on one or the other of the two Ra5 species; significant differences in ab levels to Ra5A and Ra5B were consistently found in such persons by direct binding RIA experiments (arrows in Fig. 7). These "atypical" individuals may represent 10 to 15% of the Ra5-sensitive population. Usually the binding of Ra5A to the antibodies of these persons was greater than that of Ra5B. Of particular interest, the same significant trends toward differential binding of radiolabeled Ra5A and Ra5B was found in three of four of these exceptional patients in IgE as well as IgG ab. These results are consistent with the IgG and IgE ab of a given individual recognizing the same arrays of determinants. In inhibition experiments, the binding of ^{125}I -Ra5A to the IgG and IgE ab of five allergic patients was equally well inhibited by unlabeled Ra5A and Ra5B (see examples in Figs. 8 and 9). Hyposensitization treatment did not change the inhibition patterns for IgG ab binding.

We conclude that Ra5A and Ra5B behave as indistinguishable isoantigens and isoallergens in most, but not all, Ra5-positive subjects. Amino-terminal sequencing of 30 residues revealed no differences between Ra5A and B. We conclude, therefore, that the amino acid difference(s) accounting for the charge difference and minor immunologic differences resides in the 15 C-terminal residues. Further structural studies (Klapper *et al.*, unpublished) have confirmed the sequence of Ra5A as published (8),⁵ but the complete sequence of Ra5B is not yet available.

For the genetic studies in our laboratory (11, 12) we required an Ra5 preparation of ultrapurity. As little as 1% of a potent

⁴ Patient J.Ge. (like other persons in group 3) made no detectable IgG or IgE ab before treatment and produced only an IgG ab response to Ra5 after treatment with ragweed (12).

⁵ In addition, the further variant of Ra5 (7), which differs from Ra5A by an isoleucine at position 2, has been isolated and sequenced.

allergenic contaminant in an Ra5 preparation used for intradermal skin testing could cause false-positive reactions at 10^{-3} to 10^{-4} $\mu\text{g}/\text{ml}$ in a few patients who are highly sensitive to the contaminant (8-mm wheal reactions at 10^{-5} to 10^{-6} $\mu\text{g}/\text{ml}$). This would consequently lead to false conclusions in the interpretation of skin-test data. We also recognized that antigenic contamination of 1% could lead to similar (although less severe) problems in the RIA of IgE and IgG ab. Løwenstein *et al.* (20) reported CIE and crossed-line immunoelectrophoresis (CLIE) studies of the purity of three Ra5 preparations using amounts of 10 to 50 μg . Only one preparation (Ra5-3), which had been obtained after a final purification step using high-voltage paper electrophoresis, was free from visible contaminants. Unlike the present procedure, high-voltage electrophoresis would not be applicable for large-scale preparation of Ra5. We therefore attempted to attach a numerical estimate on the degree of purity of our sample of Ra5A, the variant obtained in sufficiently large amounts to be useful in further studies.

A 100- μg load of Ra5A applied to slots in SDS-PAGE gels failed to reveal any component of different size in the preparation. This indicates at least 98% purity according to apparent m.w., because 2 μg of a protein contaminant could be detected in our SDS-PAGE system. In more discriminating CIE experiments, we could not detect any contaminants when loads of up to 60 μg of Ra5A were tested against our potent anti-RW (16-min) ab pool (Fig. 6B). On the other hand, 0.05 to 0.1 μg of a protein antigen could be detected in our CIE system. We conclude that the Ra5A preparation has an overall purity of 99.5% as an absolutely minimal estimate and is most probably $\geq 99.9\%$ pure. This clearly qualifies this material for use in the ongoing genetic studies in our laboratory.

While less stringent assays of purity were carried out with Ra5B due to the low yield, all tests showed no detectable contamination with non-Ra5 components when optimal concentrations of Ra5B were used. The presence of about 3% of Ra5A in the Ra5B preparation is explained by the similarity in the size of the two proteins (shown by SDS-PAGE) and by the fact that the difference in charge is less pronounced at pH 7.0 (the CM-Sephadex buffer pH) than at pH 8.5 (buffer pH for agarose electrophoresis). Such a low level of cross-contamination of Ra5A in Ra5B and the undetectable ($< 1\%$) contamination of Ra5B in Ra5A would have had no measurable effect on the RIA studies, which are subject to a variation of $\pm 10\%$.

Further structural studies of Ra5B are currently in progress. The identification of a few antisera capable of discriminating

between Ra5A and B will allow us to pursue rather detailed structure-function studies of antigenic/allergenic determinants on these Ra5 molecules.

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