

Reactivity of ragweed allergens with IgE antibodies

Analyses by leukocyte histamine release and the radioallergosorbent test and determination of cross-reactivity

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Five distinct proteins with allergenic activity have been isolated from short ragweed pollen. We initially tested three of these, AgE, AgK, and Ra3, for reactivity with IgE antibodies by leukocyte histamine release and by the radioallergosorbent test (RAST). We found highly significant correlations between the reactivities of these allergens by leukocyte histamine release and by the RAST, consistent with the view that both procedures detected comparable allergenic activity. We next tested the allergenic cross-reactivity of all five ragweed allergens, AgE, AgK, Ra3, Ra4, and Ra5, by RAST inhibition. With solid-phase AgE the only nonhomologous inhibitor was AgK, which cross-reacted weakly and required a 140-fold mass excess of AgK compared to AgE. With solid-phase AgK both AgK and AgE produced significant inhibition; AgE was slightly more potent than the homologous AgK. Ra3 and Ra5 were allergenically unique, because only the homologous allergen produced 50% inhibition. Ra4 was weakly inhibited by AgE, Ra3, and Ra5 when these allergens were added in 300- to 500-fold mass excesses; this weak inhibition may represent either cross-reaction or cross-contamination. We found that RAST inhibition could be used as an assay for the individual ragweed allergens and we demonstrated the presence of all of the allergens in a whole ragweed extract. The sensitivity of the RAST inhibition assay ranged from 10 ng to 100 ng for 50% inhibition. Finally, the solid-phase ragweed allergens were used to determine the frequency of elevated IgE antibody levels in 65 patients with ragweed hay fever. Virtually all of the patients reacted with AgE (97%), while 88% reacted with AgK, 51% reacted with Ra3, 28% reacted with Ra4, and 17% reacted with Ra5. These results highlight the usefulness of the RAST as a specific and sensitive tool for immunochemical studies of allergens.

Since 1962 five allergens have been isolated from short ragweed pollen: AgE, AgK, Ra3, BPA-R (Ra4), and Ra5 (recently reviewed by King¹). These proteins were identified by their ability to provoke wheal-and-flare skin reactions in humans and were

subsequently investigated with animal antisera to establish their antigenic cross-reactivity. Antigenic partial identity was observed between AgE and AgK² and between AgE and Ra4³ with rabbit antisera to crude ragweed pollen. No evidence of antigenic cross-reactivity was observed between AgE and Ra3⁴; Ra3 and Ra4³; and Ra5 and AgE, Ra3, and Ra4.⁵ Extensive allergenic cross-reactivity has been documented between AgE and AgK by direct skin tests and by histamine release from leukocytes.² By the same procedures AgE and Ra3 have been shown to be allergenically distinct.⁶ Thus, the results from the literature suggest that antigenic and allergenic cross-reactivity exists between AgE and AgK and antigenic cross-reactivity exists between AgE and Ra4, while Ra3 and Ra5 are distinct antigens which do not cross-react.

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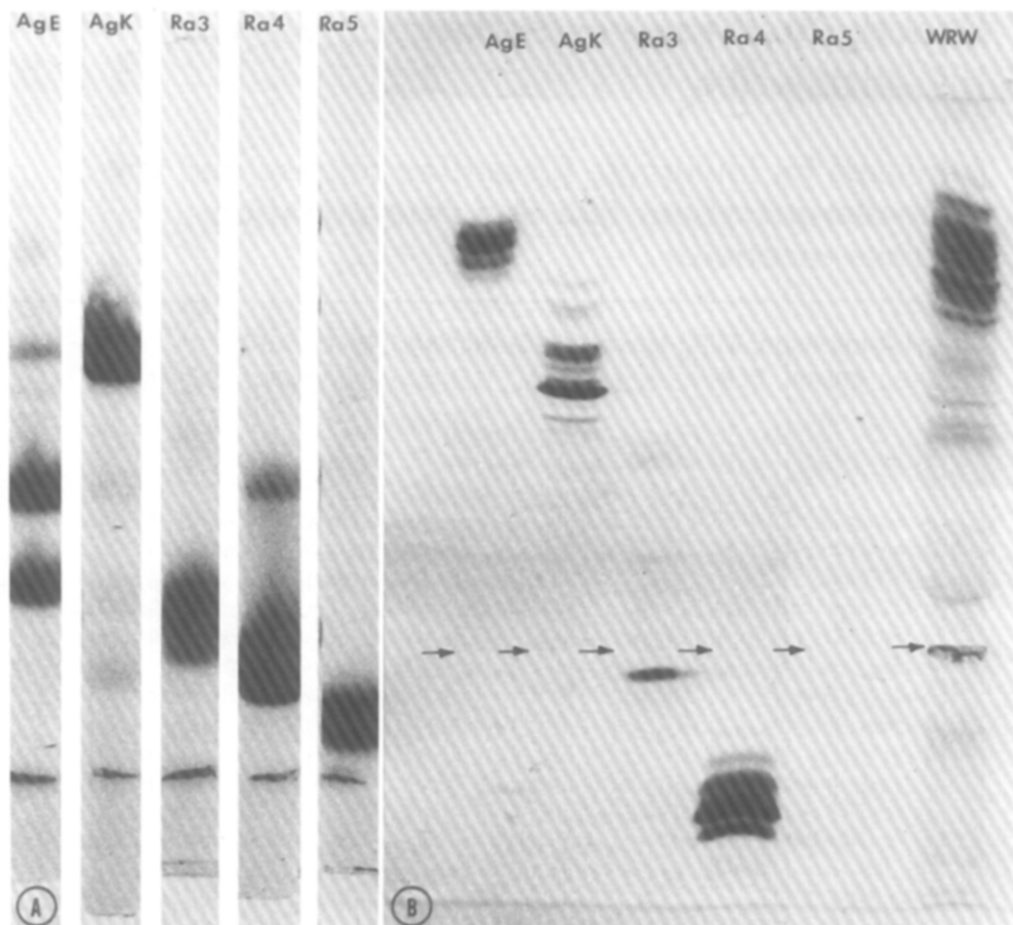


FIG. 1. *A*, In the polyacrylamide gels equilibrated with sodium dodecylsulfate the proteins were reduced with dithiothreitol and stained with Coomassie blue. Marker proteins used were: BSA, 6.9×10^4 daltons; ovalbumin, 4.5×10^4 daltons; chymotrypsinogen, 2.4×10^4 daltons; and cytochrome C, 1.2×10^4 daltons. The regression line for the marker proteins had a correlation coefficient of -0.999 . The origin is at the top of each gel and the anode is at the bottom of the figure. From left to right, the samples, amounts applied to each gel, calculated and literature¹ molecular weights (in daltons) are: AgE, 10 μ g, 2.3×10^4 and 1.5×10^4 (major bands), 3.9×10^4 (minor band), 2.6×10^4 and 1.3×10^4 (literature); AgK, 14 μ g, 4.0×10^4 , 3.8×10^4 (literature); Ra3, 10 μ g, 1.3×10^4 (major band), 1.2×10^4 (minor band), 1.1×10^4 (literature); Ra4, 50 μ g, $<1.2 \times 10^4$ (major band), 2.3×10^4 (minor band), 2.3×10^4 (literature); Ra5, 10 μ g, $<1.2 \times 10^4$, 5.0×10^3 (literature). Because of the large sample loads used to check heterogeneity, the molecular weight determinations were accurate only to 20% of the expected values. *B*, In the electrofocusing in a polyacrylamide gel slab with a pH range of 3 to 10, the gel was stained with Coomassie blue. The anode (pH 3) is at the top; the origin for each sample is indicated by an arrow; the samples and amounts applied to the gel from left to right are: AgE, 30 μ g; AgK, 38 μ g; Ra3, 25 μ g; Ra4, 70 μ g; Ra5, 30 μ g; and WRW extract, 200 μ g.

To investigate the allergenic properties of the ragweed antigens, we utilized the radioallergosorbent test (RAST).⁷ We compared leukocyte histamine release with the purified allergens AgE, AgK, and Ra3 to the level of IgE antibody as determined by the RAST. RAST inhibition⁸ was used in this study to evaluate the allergenic cross-reactivity and cross-contamination of the five allergens. Finally, sera from 65 patients with ragweed hay fever containing elevated levels of IgE antibodies to whole ragweed pol-

len were assayed for their reactivities with the individual purified allergens.

MATERIALS AND METHODS

Purified short ragweed antigens

Antigen E (Catalogue No. A601-902-185), antigen K (Catalogue No. A602-901-185), and ragweed Ra3 (Catalogue No. A604-902-185) were supplied by Dr. J. Nutter, Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health,

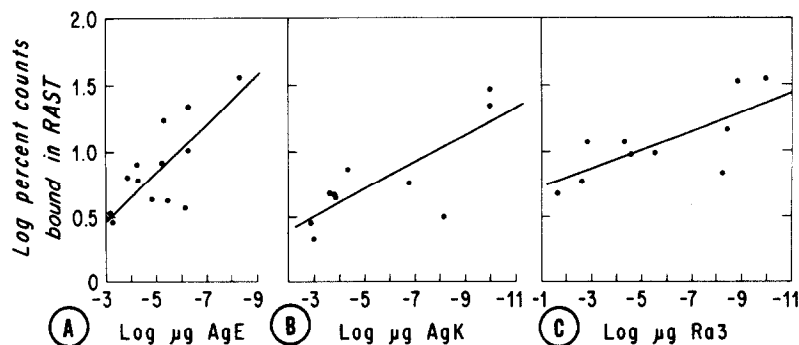


FIG. 2. Comparison of RAST and histamine release from leukocytes for the ragweed allergens, AgE, AgK, and Ra3. The solid-phase allergen utilized for *A* was AgE, for *B* was AgK, and for *C* was Ra3. In each graph the log of the percent counts bound for the solid-phase allergen is plotted on the ordinate and the log of the μg of allergen required for 50% histamine release is plotted on the abscissa. The serum used for the RAST was drawn the same day as the leukocytes were obtained from each patient.

Bethesda, Md. Ragweed antigens Ra4 and Ra5 were prepared as described previously.^{3, 5, 9-11} All purified antigen concentrations were determined by previously published extinction coefficients with the exception of Ra4, which was assumed to have $E_{1\text{ cm}}^{0.1\%} = 1.0$ at 280 nm. Analyses of these

proteins by polyacrylamide gels equilibrated with 1% sodium dodecylsulfate¹² and by electrofocusing in a polyacrylamide gel slab¹³ are shown in Fig. 1. In general, the antigen preparations show a high degree of homogeneity. On the sodium dodecylsulfate electrophoresis in the presence of dithiothreitol (Fig. 1, *A*), some extraneous components are evident at the large sample loads employed. For example, the AgE sample has a very faint band with the same migration distance as the AgK band. It is noteworthy that AgK and Ra3 are single-chain proteins. With the exception of AgK, which shows a few very faint bands with the same isoelectric points as some of the AgE bands, the electrofocusing results shown in Fig. 1, *B* further illustrate the homogeneity of these antigens. Interestingly, Ra3 has a single isoelectric form while Ra4 has several. No bands are visible for Ra5, because it migrated into the cathodal wick. Taken together, these two techniques show the virtual lack of cross-contamination among the samples.

Short ragweed pollen extract

Preparation of the short ragweed pollen extract (WRW) has been previously described.¹⁴ Briefly, a lyophilized extract of short ragweed pollen (WRW) was prepared by exposing the pollen (not diethyl ether defatted) to distilled water for 48 hr. The extract was filtered, centrifuged, concentrated, and sieved through Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N. J.); the partially purified extract was lyophilized and stored at 4°C. Dry weight of the WRW extract was determined by dissolving the lyophilized powder in 0.2 M H_3BO_3 -0.04 M NaOH-0.16 M NaCl, pH 8.0 (borate-saline), centrifuging the solution, and drying a known volume to constant weight in a 110°C oven. Similarly, a sample of the borate-saline was also dried and its mass subtracted.

TABLE I. IgE antibody levels with ragweed allergens: Profiles of the serum pools

Serum	Solid-phase allergen					
	AgE	AgK	Ra3	Ra4	Ra5	WRW
1	30.60*	18.74	20.86	2.55	1.12	31.88
2	22.57	14.10	14.26	1.54	1.02	22.42
3	22.85	14.26	7.79	3.20	2.79	23.06
4	27.96	20.82	10.35	1.04	14.11	22.60
5	25.23	21.01	19.45	4.29	35.67	37.91
AgE, AgK, Ra3, Ra5, and WRW pool	19.67	16.86	12.12	1.16	14.60	33.80
Normal	0.61	0.61	0.56	0.60	0.70	0.42
6	10.60	5.97	2.00	3.83	0.85	14.19
7	8.64	4.58	2.49	2.78	0.76	17.39
8	21.69	13.85	10.52	5.48	1.08	28.92
Ra4 pool	—†	—	—	4.72	—	—
Normal	0.61	0.61	0.56	0.60	0.70	0.42

*Tabulated values are percent of total counts bound in the RAST (counts bound/counts added) $\times$ 100.

†Not determined.

Solid-phase short ragweed pollen

As described earlier,¹⁴ the solid-phase whole ragweed pollen extract was prepared by the reaction of the WRW prepared above with cyanogen bromide (CNBr)-activated cellulose. The details of the coupling procedure have been described previously.¹⁵

Solid-phase purified antigens

Two separate batches of solid-phase antigens were prepared for this study. First, 1 mg of antigen was reacted with 500 mg of cellulose and, second, 1 or 2 mg of antigen plus 9

mg of human serum albumin* (HSA, Schwarz/Mann, Orangeburg, N. Y.) were reacted with 450 mg of cellulose. Both preparations reacted with IgE antibodies in a comparably sensitive manner when titrated in the RAST.[†]

The solid-phase AgE, AgK, and Ra3 antigens used in the patient survey and histamine release correlation were prepared by reaction of antigen only (no HSA) with CNBr-activated cellulose as described previously.¹⁵ Briefly, 1.5 gm of microcrystalline cellulose was activated at pH 11 by reaction with 3.0 gm of CNBr dissolved in 3.0 ml of N, N-dimethylformamide. The activated cellulose was washed twice with borate-saline and divided into three aliquots. Each aliquot was reacted with 1.0 mg of antigen suspended in a volume of 10 ml in borate-saline and the pH adjusted to 8.3. The suspension was tumbled at 4° C for 6 days before it was repeatedly washed with borate-saline until the absorbance at 280 nm was less than 0.015. By monitoring the absorbance at 280 nm and the volumes of the coupling supernatant and the borate-saline washings, no more than 0.50 mg AgE, 0.47 mg AgK, and 0.22 mg Ra3 were coupled to 500 mg cellulose.

The five solid-phase antigens used in the inhibition studies as well as the Ra4 and Ra5 used in the patient survey were prepared with HSA added as an inert filler protein. One-mg portions of AgE, AgK, Ra3, and Ra5, and 2.0 mg of Ra4 were separately combined with 450-mg portions of washed, freshly activated cellulose. Immediately after the antigens were added to the activated cellulose, 9.0 mg of HSA was added to each portion of cellulose. The volume of

each suspension was brought to 11 ml with borate-saline, pH 8.1, and the suspensions were tumbled at 4° C for 72 hr. The cellulose was repeatedly washed with borate-saline until the absorbance at 280 nm was zero. The quantities of proteins bound to the cellulose ranged between 71.1% and 78.3%. When the supernatants from each coupling suspension were tested by RAST inhibition (*vide infra*) to detect uncoupled antigen, no inhibition was produced in any of the homologous systems.

Source of IgE antibodies

The patients whose sera were used in the survey and the histamine release study have been described previously.¹⁸ The 65 patients in this study were chosen on the basis of: past history of seasonal rhinitis, positive immediate intradermal skin test, and positive nasal challenge with crude short ragweed extract and an elevated preseasonal (June) level of serum IgE antibodies (percent total counts bound >7 × control) to short ragweed as measured by the RAST. The sera used were post-pollination season (October) samples that had been stored at -20° C.

The ragweed serum pool used for the RAST inhibition procedures, with the exception of solid-phase Ra4 (*vide infra*), has been described elsewhere.¹⁴ This pool consisted of equal volumes of serum from 5 non-hyposensitized subjects, who were highly allergic to short ragweed pollen. The pool contained 2,370 ng/ml of IgE antibody to WRW extract¹⁴ as determined by an immunoabsorption technique.¹⁹ As shown in Table I, each serum showed elevated levels of IgE antibodies (percent total counts bound >14 × control) to solid-phase AgE, AgK, and Ra3. Two of the five sera showed elevated levels of IgE antibodies (percent total counts bound >20 × control) to solid-phase Ra5. For the Ra4 inhibition study, a different serum pool was selected on the basis of the patient survey (*vide infra*, Table III). Three patients were selected whose sera showed elevated levels of IgE antibodies (percent total counts bound >4 × control) to solid-phase Ra4.

Performance of the RAST

Serum IgE antibodies to ragweed pollen allergens were measured by RAST essentially as described previously.¹⁵ Briefly, 0.50 mg solid-phase ragweed allergen, suspended in 0.50 ml RAST diluent (0.1 M KH_2PO_4 - K_2HPO_4 , pH 7.4, 0.2% bovine serum albumin [BSA], 1% Tween-20, and 0.1% sodium azide), was added to 0.050 ml patient's serum and brought to a final volume of 0.75 ml with RAST diluent in 10 × 62 mm tubes (Luckham Ltd., Sussex, England). The tubes were capped, rotated slowly overnight at room temperature, and centrifuged; the supernatant was aspirated; and the solid-phase allergen was washed twice with RAST wash (0.1 M KH_2PO_4 - K_2HPO_4 , pH 7.4, and 1% Tween-20). Fetal calf serum (FCS) diluent, 0.60 ml (0.1 M KH_2PO_4 - K_2HPO_4 , pH 7.4, 470 ml; FCS purchased from Grand Island Biologicals, Grand Island, N. Y., 20 ml; 10% NaN_3 , 5 ml; and Tween-20, 5 ml), was added to each tube followed by 20 ng of radiolabeled affinity chromatography-purified anti-IgE in 0.10 ml of phosphate albumin (0.1 M KH_2PO_4 - K_2HPO_4 , pH 7.4, and 1% BSA). The tubes were

*An earlier report¹⁶ compared the solid-phase materials produced with varying amounts of crude ragweed extract reacted with activated cellulose. When tested in the RAST, the solid-phase material prepared with a mass ratio of 100 mg crude extract to 500 mg cellulose had both a greater slope of its serum pool (ragweed-allergic) dilution curve and a smaller nonspecific binding with normal serum than the solid-phase material prepared with a mass ratio of 1 mg crude extract to 500 mg cellulose. Similar observations¹⁷ have been made with the purified rye grass pollen antigen, Group I. When 0.1, 1.0, and 10 mg of Group I were reacted with 500-mg portions of cellulose, there was a direct correlation between the larger mass of allergen and the greater slope of the serum pool (rye grass-allergic) dilution curve. In addition, the nonspecific binding was reduced with increased allergen coupled to the cellulose. In order to conserve purified allergen, we also tested 1.0 mg of Group I and 9.0 mg of HSA reacted with 500 mg of cellulose. The inclusion of HSA produced a solid-phase material with virtually the same slope of the serum dilution curve and the same nonspecific binding as the material with 10 mg of Group I reacted with 500 mg of cellulose. Although the mechanism for this increased sensitivity was not studied, we suspect that the HSA acts as a blocking protein on the activated cellulose and possibly reduces the nonspecific adsorption of IgE and/or anti-IgE to the cellulose.

†In contrast to the rye grass pollen Group I solid-phase materials, the AgE, AgE and HSA; AgK, AgK and HSA; Ra3, Ra3 and HSA solid-phase materials produced parallel serum pool (ragweed-allergic) dilution curves and had comparable levels of nonspecific binding with normal serum. The reasons for this different behavior of the ragweed allergens and the rye grass allergen are obscure.

TABLE II. Comparison between RAST and histamine release by leukocytes*

Patient	RAST AgE	Histamine release with AgE (log μ g)	RAST AgK	Histamine release with AgK (log μ g)	RAST Ra3	Histamine release with Ra3 (log μ g)
128	36.25	-8.34	29.21	<-10	34.39	<-10
108	21.74	-6.29	21.66	<-10	32.67	-8.82
106	10.73	-6.29	5.67	-6.77	14.49	-8.46
159†	3.75	-6.14	2.77	>-2.85	9.52	-4.60
151	4.31	-5.48	3.18	-8.13	6.56	-8.24
100	17.80	-5.39	—‡	—	—	—
113	8.30	-5.22	7.08	-4.38	0.44	>-0.78
159†	4.24	-4.85	—	—	—	—
112	5.97	-4.28	4.74	-3.65	9.63	-5.58
103	8.04	-4.25	4.62	-3.84	11.86	-4.35
105	6.25	-3.84	4.32	-3.85	11.64	-2.82
123	2.91	-3.27	2.84	-2.90	4.71	-1.70
135	3.40	-3.18	2.14	-2.96	5.82	-2.64
Normal	1.35	None§	1.41	None	1.34	None

*The RAST with solid-phase AgE and AgK was performed using 5 μ l of each patient's serum; solid-phase Ra3 utilized 50 μ l of serum. The nonspecific histamine release ranged from 0.0% to 19.5% of total histamine available and the average was 4.7%. RAST results are expressed as (counts bound/counts added) $\times$ 100; histamine release results are expressed as the log μ g of allergen to provoke 50% release in 1.0 ml of cell suspension. Patient No. 100 met the criteria for the patient survey population but is not included in Table III.

†This patient provided plasma on two different dates.

‡Not determined.

§No histamine released by any allergen concentration up to 10^{-1} μ g/ml.

rotated overnight and washed twice before the radioactive counts associated with the solid-phase allergen were determined on a gamma spectrometer. The results are given as (counts bound divided by counts added) $\times$ 100%.

RAST inhibition

The RAST inhibition procedure was carried out essentially as described previously.^{8, 15} For each solid-phase allergen, the quantity of serum pool added had been established by preliminary direct RAST titration experiments as that volume of serum pool providing at least $10\times$ the counts bound by normal human serum. The equivalent volumes of undiluted serum pool used with each solid-phase system were as follows: whole ragweed, 2 μ l; AgE and AgK, 5 μ l; Ra3, 20 μ l; Ra5, 10 μ l; and Ra4, 100 μ l (of the Ra4 pool). Preliminary inhibition experiments were carried out over a 100,000-fold range of dilutions of inhibitor to determine a narrow mass range for inhibitor which would provide approximately 50% inhibition. Briefly, inhibitor, diluted in RAST diluent, was added to each tube; additional RAST diluent was added as necessary before quickly adding allergic serum pool to each tube. The 0.25-ml volume in each tube was briefly mixed before the rapid addition of 0.50 ml of solid-phase ragweed allergen at a cellulose concentration of 1.0 mg per ml. After the tubes were rotated for 20 hr at room temperature, they were washed twice and anti-IgE and FCS diluent were added. After overnight rotation, the solid-phase allergen was washed twice and the radioactivity

in the solid phase determined in a gamma spectrometer. Each inhibition point was determined in duplicate; each uninhibited positive control was determined in quadruplicate; the results were averaged and the percent of total counts bound by the normal serum control was subtracted from all of the tests.

Each allergen was tested as a fluid-phase inhibitor with every solid-phase allergen. Nonparallel inhibition lines were taken as evidence of allergenic cross-reactivity^{8, 20, 21} and parallel inhibition curves as evidence for qualitative allergenic similarity.²² Those allergens that did not inhibit significantly in preliminary experiments were included in the final inhibition at two or more high concentrations.

Leukocyte histamine release

The histamine release from leukocytes was carried out essentially as previously described²³ and modified.²⁴ Briefly, after sedimentation and centrifugation the unwashed leukocyte sediment was resuspended to a volume equal to the original volume of blood. The cells were challenged with antigen by incubating 1.0-ml aliquots of the suspension, 0.10 ml antigen dilutions, and 0.10 ml of AB normal serum at 37°C for 1 hr. The histamine released was sequentially extracted into 1-butanol and n-heptane-0.12 M HCl before being detected by the *o*-phthaldialdehyde reaction. Relative intensity fluorescence readings were taken on an Aminco-Bowman spectrophotofluorometer (American In-

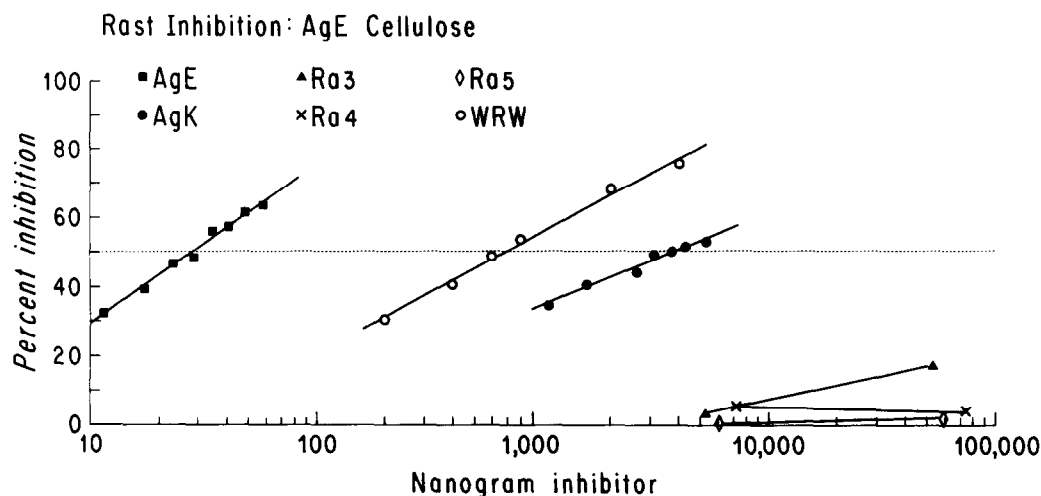


FIG. 3. Inhibition curves showing the cross-reactivity of the purified ragweed allergens and whole ragweed pollen extract with solid-phase AgE. In this and subsequent inhibition figures, the log-linear plots provided curves which were linear between 30% and 70% inhibition. For AgE, AgK, and WRW, the regression lines relating percent inhibition and log mass inhibitor had correlation coefficients of +0.99 or greater. By analysis of covariance, the slopes of these three regression lines differed ($F_{2,15} = 21$, $p < 0.0005$). In the absence of inhibitor the serum pool IgE antibody produced 16.86% total counts bound and the normal serum produced 0.99% total counts bound.

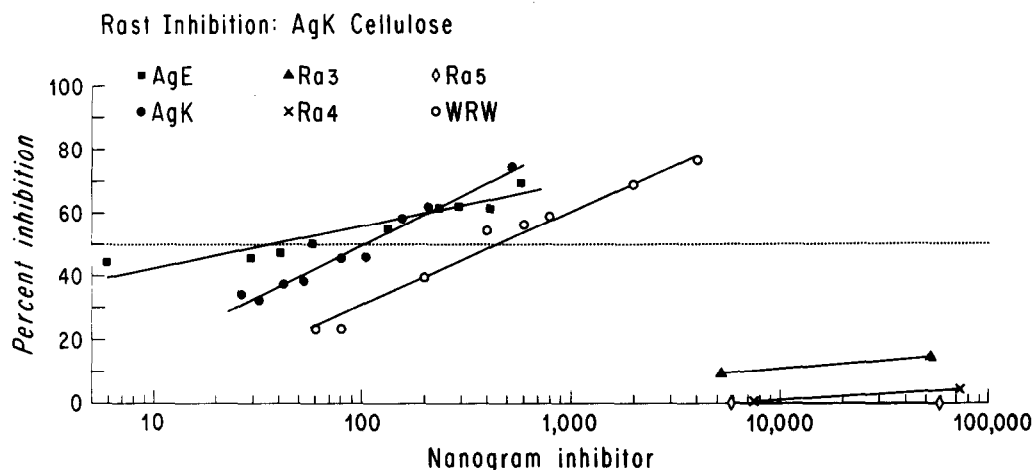


FIG. 4. Inhibition curves to examine the cross-reactivity of the purified ragweed allergens and whole ragweed pollen extract with solid-phase AgK. The regression lines relating percent inhibition and mass inhibitor had correlation coefficients of +0.99 for AgK and WRW and +0.94 for AgE. By analysis of covariance the slopes of the three regression lines differed ($F_{2,20} = 36$, $p < 0.0005$). In the absence of inhibitor the serum pool IgE antibody produced 8.94% total counts bound and the normal serum produced 0.79% total counts bound.

strument Co., Silver Spring, Md.) with excitation at 360 nm and emission at 450 nm. All the assays included internal and external histamine-HCl standards.

Statistical analyses

Results were evaluated by analysis of covariance and Spearman's rank correlation coefficients.²⁵ A programmable Hewlett-Packard 9810A calculator was used for the statistical evaluations.

RESULTS

Histamine release correlation with RAST

Inspection of the results in Table II and Fig. 2 suggests that a larger quantity of IgE antibody as determined by the RAST corresponds to a smaller mass of allergen required for 50% histamine release. To investigate whether there was a significant correlation between the mass of allergen needed for 50% his-

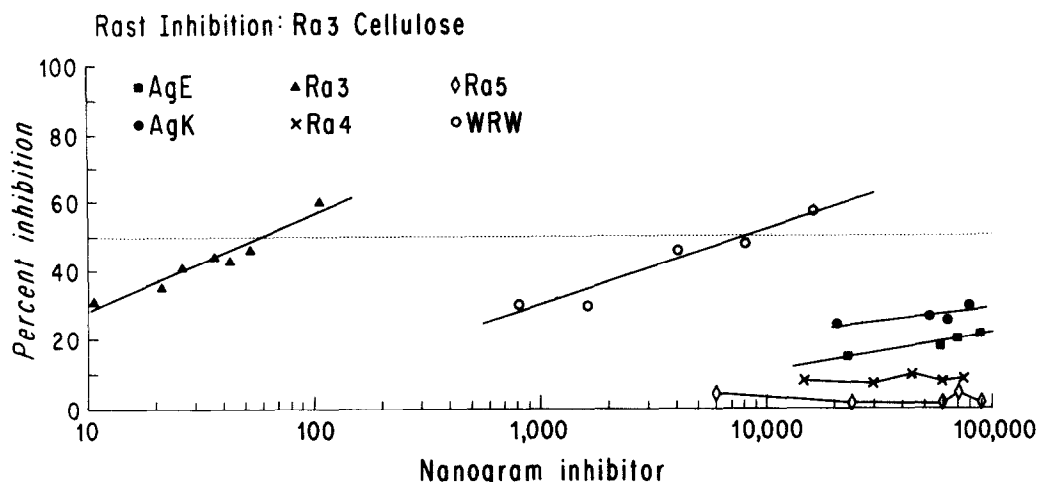


FIG. 5. Inhibition curves to examine the cross-reactivity of the purified ragweed allergens and whole ragweed pollen extract with solid-phase Ra3. The regression lines relating percent inhibition and mass inhibitor for Ra3 and WRW had correlation coefficients of +0.966. In the absence of inhibitor the serum pool IgE antibody produced 14.28% total counts bound and the normal serum produced 0.73% total counts bound.

tamine release and the magnitude of the RAST results, the data in Table II were analyzed by the Spearman rank test. The rank analysis indicated a significant correlation for all three allergen systems with correlation coefficients as follows: AgE, $r_s = 0.676$, $p < 0.01$; AgK, $r_s = 0.791$, $p < 0.005$; and Ra3, $r_s = 0.836$, $p < 0.005$.

RAST inhibition studies of allergenic specificity

To examine the allergenic cross-reactivity and possible cross-contamination of the ragweed allergens, RAST inhibition studies were performed. Fig. 3 shows the RAST inhibition results for solid-phase AgE. Fluid-phase AgE produced 50% inhibition with 28 ng of allergen while the WRW extract required 690 ng for 50% inhibition and AgK required 3,900 ng for 50% inhibition. The slopes of the inhibition lines for AgE and AgK were statistically different ($F_{1,11} = 49$; $p < 0.0005$), in agreement with earlier reports of the cross-reactivity of AgK with AgE.² If there were also cross-contamination between AgE and AgK, the contamination would have been less than 28 parts in 3,900 parts or 0.72% by mass. The slopes of the inhibition lines for AgE and WRW were also statistically different ($F_{1,10} = 15$; $p < 0.005$). None of the basic protein allergens, Ra3, Ra4, and Ra5, were able to inhibit the solid-phase AgE.

The RAST inhibition study for solid-phase AgK is shown in Fig. 4. Fluid-phase AgK produced 50% inhibition with 103 ng while AgE required 38 ng for 50% inhibition and WRW extract required 450 ng for

50% inhibition. The slopes of the regression lines for AgK and WRW extract did not differ statistically ($F_{1,13} = 0.83$, $p > 0.25$), but AgE had a significantly lower slope compared to both AgK ($F_{1,14} = 49$, $p < 0.0005$) and WRW extract ($F_{1,13} = 45$, $p < 0.0005$). Thus, AgE cross-reacted extensively with AgK and significant allergenic similarity existed between AgK and this particular preparation of WRW extract. As was the case with solid-phase AgE, none of the basic protein allergens, Ra3, Ra4, and Ra5, were able to inhibit the solid-phase AgK.

Fig. 5 shows the RAST inhibition study for solid-phase Ra3. In this study, Ra3 was allergenically unique because significant inhibition occurred only with the homologous allergen and with the WRW extract. Fluid-phase Ra3 produced 50% inhibition with 60 ng and WRW extract required 8,400 ng for 50% inhibition. The slopes of the regression lines for Ra3 and WRW extract did not differ statistically ($F_{1,8} = 1.63$, $p > 0.10$), indicating significant allergenic similarity between Ra3 and this particular WRW extract. Both fluid-phase AgE and AgK provided weak inhibition at high concentrations. By inspection, the slopes of the inhibition lines for fluid-phase AgE and AgK were less than the slope of the inhibition line for fluid-phase Ra3. Neither Ra4 nor Ra5 provided any inhibition of the solid-phase Ra3, which indicated a lack of cross-reactivity or contamination.

The RAST inhibition results for solid-phase Ra4 are shown in Fig. 6. Fluid-phase Ra4 was the most potent inhibitor, requiring 100 ng for 50% inhibition,

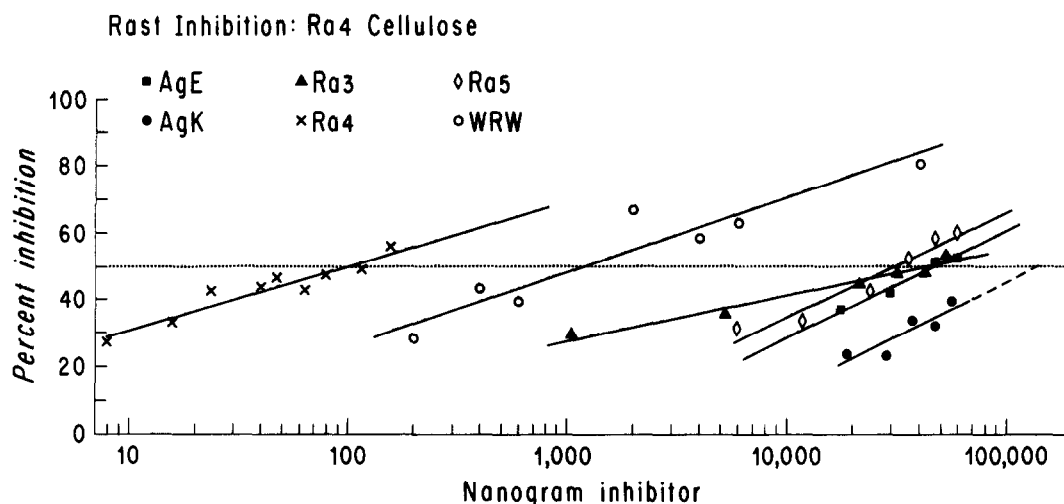


FIG. 6. Inhibition curves to examine the cross-reactivity of the purified ragweed allergens and whole ragweed pollen extract with solid-phase Ra4. The regression lines relating percent inhibition and mass inhibitor had correlation coefficients of +0.95 or greater with the exception of AgK which had a coefficient of +0.89. By analysis of covariance the slopes of the six regression lines differed ($F_{5,24} = 3.07$, $p < 0.005$). In the absence of inhibitor the Ra4 serum pool IgE antibody produced 4.73% total counts bound and the normal serum produced 0.60% total counts bound.

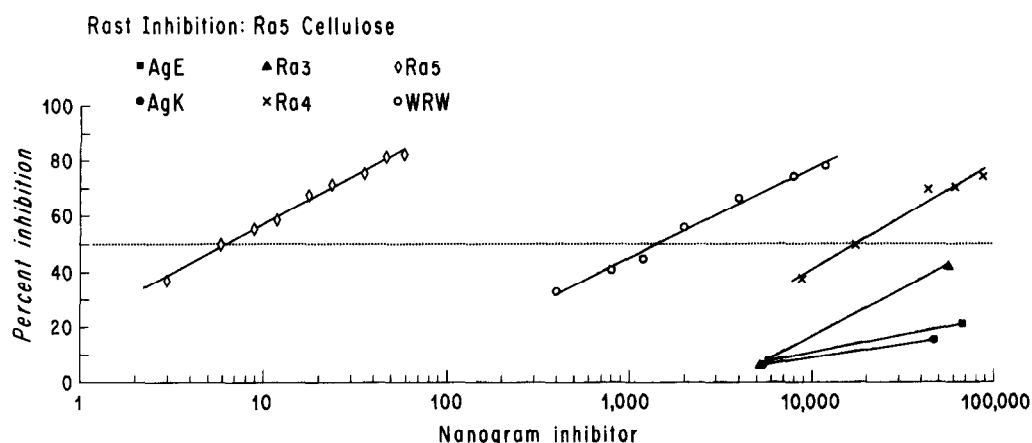


FIG. 7. Inhibition curves to examine the cross-reactivity of the purified ragweed allergens and whole ragweed pollen extract with solid-phase Ra5. The regression lines relating percent inhibition and mass inhibitor had correlation coefficients of +0.988 or greater. By analysis of covariance the slopes of the regression lines for Ra5, Ra4, and WRW were not different ($F_{2,15} = 2.64$, $p > 0.1$). In the absence of inhibitor the serum pool IgE antibody produced 7.45% total counts bound and the normal serum produced 0.45% total counts bound.

while WRW extract required 1,200 ng, Ra5 required 31,000 ng, Ra3 required 43,000 ng, and AgE required 46,000 ng. Extrapolation of the AgK inhibition line suggested that 138,000 ng would be required for 50% inhibition. The slopes of the regression lines for Ra4 and WRW did not differ statistically ($F_{1,11} = 0.38$; $p > 0.5$); the slopes of the regression lines for AgE, AgK, and Ra5 did not differ statistically ($F_{2,9} = 0.0148$; $p > 0.975$).

Fig. 7 shows the RAST inhibition study for Ra5. The homologous fluid-phase allergen required 6.4 ng for 50% inhibition while the WRW extract required

1,450 ng for 50% inhibition and Ra4 required 18,000 ng for 50% inhibition. The slopes of the regression lines for Ra5 and Ra4 did not differ statistically ($F_{1,10} = 1.22$, $p > 0.25$); likewise, the slopes for Ra5 and the WRW extract did not differ statistically ($F_{1,12} = 2.88$, $p > 0.10$). Fluid-phase AgE and AgK did not inhibit solid-phase Ra5 and at 56,000 ng Ra3 provided 43% inhibition. Because the slopes of the inhibition lines for fluid-phase Ra5 and Ra4 did not differ significantly and because the serum pool used in this inhibition had very low levels of IgE antibodies for Ra4 (Table I), the inhibition observed for Ra4 was

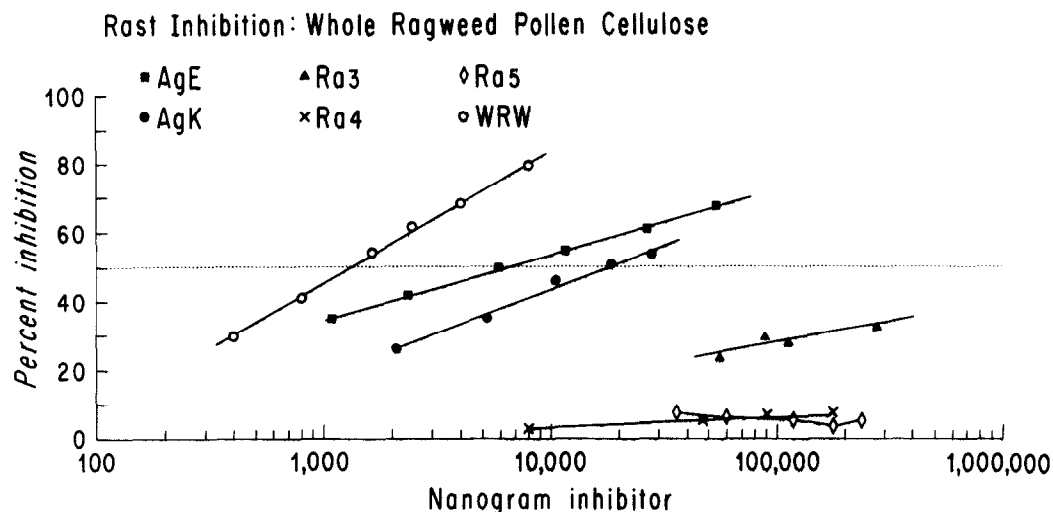


FIG. 8. Inhibition curves to examine the cross-reactivity and inhibitory capacities of the purified ragweed allergens and whole ragweed pollen extract with solid-phase whole ragweed. The regression lines relating percent inhibition and mass inhibitor for whole ragweed extract, AgE, and AgK had correlation coefficients of +0.99 or greater. By analysis of covariance the slopes of the regression lines differed ($F_{2, 11} = 110$, $p < 0.0005$). In the absence of inhibitor the serum pool IgE antibody produced 13.77% total counts bound and the normal serum produced 0.20% total counts bound.

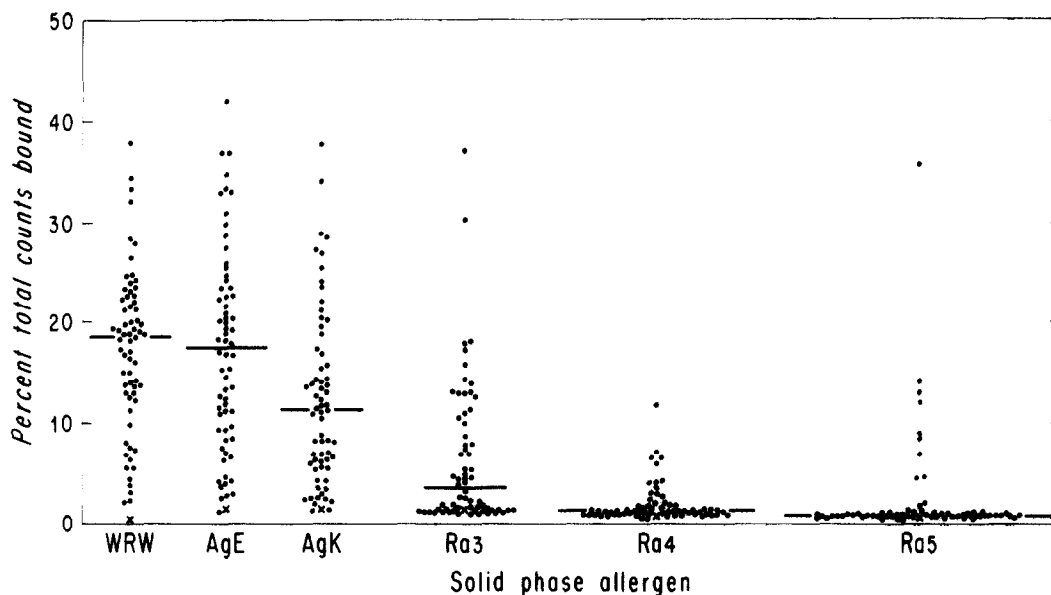


FIG. 9. Patient survey distribution by RAST for the five allergens and whole ragweed pollen. The median percent total counts bound by the patients is indicated by a horizontal line and the percent total counts bound by normal serum is indicated by an X.

probably due to a trace contamination by Ra5 (0.036% by mass or 0.16% on a molar basis). If the inhibition observed for Ra3 was due to contamination by Ra5, the quantity would have been less than 0.01% by mass.

The RAST inhibition results for the solid-phase whole ragweed pollen extract are shown in Fig. 8. Fluid-phase whole ragweed pollen extract required

1,300 ng for 50% inhibition while AgE required 6,600 ng and AgK required 19,000 ng for 50% inhibition. Fluid-phase Ra3 appeared to inhibit slightly at very high concentrations and Ra4 and Ra5 showed no inhibitory properties. The slope of the regression line for the fluid-phase WRW was greater than the slope for fluid-phase AgE ($F_{1,8} = 469$, $p < 0.0005$) and greater than the slope for fluid-phase AgK ($F_{1,7} =$

TABLE III. IgE antibody levels with ragweed allergens: Patient survey

Patient	Solid-phase allergen					
	WRW	AgE	AgK	Ra3	Ra4	Ra5
101	13.69*	11.78	6.36	7.82	1.12	0.84
102	16.81	18.26	8.06	1.11	0.73	0.72
103	24.05	29.91	20.41	14.30	11.77	0.81
104	28.31	28.63	24.00	17.89	7.02	1.19
105	20.09	21.55	19.51	12.85	1.25	0.72
106	26.53	32.90	23.50	18.06	2.03	0.75
107	12.64	8.39	6.59	3.84	0.41	0.42
108	33.31	36.91	34.01	30.29	4.02	1.77
109	14.09	18.15	11.05	7.33	1.02	0.77
110	2.25	1.14	1.30	1.20	0.93	0.83
111	19.09	20.31	11.32	1.49	1.04	0.74
112	23.01	24.64	18.83	10.93	2.01	0.95
113	20.15	25.46	20.27	1.52	2.95	1.34
114	4.37	4.15	2.88	0.93	0.96	0.37
115	9.78	17.93	12.29	1.56	1.05	0.77
117	23.52	22.61	12.69	12.97	1.53	0.98
118	2.99	2.89	2.52	1.40	0.82	1.00
119	23.32	33.26	26.82	15.81	1.34	11.95
120	19.11	9.58	5.66	4.40	5.92	0.91
121	5.48	6.28	5.31	2.28	1.08	1.18
122	11.20	13.35	6.87	1.20	1.16	1.31
123	16.42	16.72	14.03	6.91	1.15	4.50
124	17.17	17.01	13.33	1.44	1.56	0.76
125	24.30	34.62	28.79	1.90	0.77	0.95
126	19.25	11.19	11.10	1.24	0.90	1.20
127	2.04	2.46	1.83	1.80	0.80	1.16
128	37.91	41.99	37.77	37.16	4.29	35.67
129	24.59	32.99	28.45	2.59	1.70	0.92
130	22.64	20.85	11.71	1.89	1.28	14.16

*Tabulated values are percent of total counts bound in the RAST (counts bound/counts added) $\times$ 100.

†Indicates those patients who were receiving injections of ragweed allergens.

TABLE III. Cont'd

131†	6.35	4.11	3.45	2.57	1.35	2.10
132†	31.99	30.90	27.27	17.14	2.62	8.45
133†	18.14	12.36	8.75	8.64	1.12	0.81
134†	27.99	23.56	15.31	13.98	2.38	0.69
135	13.62	13.51	6.09	4.86	1.22	0.73
136†	24.78	12.65	8.14	4.51	6.52	1.07
137	18.74	22.49	14.38	1.39	0.86	0.73
138	6.82	4.60	2.32	1.39	1.77	0.75
139	15.00	20.32	16.89	1.00	1.10	0.73
140	22.65	15.33	10.92	12.64	1.53	8.83
141	13.01	14.51	10.43	6.92	0.86	0.85
142	18.54	16.85	11.22	4.70	1.74	0.76
143	21.56	27.40	22.03	1.25	1.28	0.77
144	5.46	6.64	2.20	1.09	0.83	0.70
145	22.27	25.86	21.18	1.07	0.77	0.78
146	21.37	22.24	14.27	9.95	6.46	0.95
147	19.80	15.26	13.76	13.20	1.31	0.52
148	13.85	10.82	6.84	4.02	1.34	1.16
149	18.32	3.67	3.51	2.17	3.46	0.86
150	7.53	3.91	2.44	1.09	0.79	0.78
151	21.39	19.41	12.97	7.91	0.84	0.74
152	7.29	7.21	5.51	1.09	3.04	0.67
153	16.02	9.30	6.02	5.29	1.57	0.67
154	18.94	19.24	8.20	10.47	0.87	0.54
155	3.82	7.04	1.28	1.08	0.45	0.75
156	34.34	36.90	25.84	1.71	1.14	0.98
157	19.30	24.18	15.78	1.25	1.12	6.94
158	17.39	2.73	2.37	1.17	4.06	4.61
159	19.93	20.10	13.87	13.08	1.81	13.01
160	13.00	11.44	6.37	11.36	1.40	0.64
161	22.01	23.44	11.70	0.95	0.93	0.76
162	18.82	11.16	8.15	4.58	1.29	0.80
163	12.22	8.17	4.22	3.30	1.41	0.78
164	14.15	18.90	7.07	1.00	0.87	0.68
166	7.99	9.30	4.23	1.23	0.90	0.91
168	15.03	19.86	17.39	5.42	0.92	0.67
Normal	0.42	1.35	1.41	1.34	0.75	0.70

41.8, $p < 0.0005$). The slope for fluid-phase AgK was greater than the slope for fluid-phase AgE ($F_{1,7} = 18.4$, $p < 0.005$).

Population survey

Having established the allergenic specificities of the solid-phase allergens, we examined sera from 65 ragweed-sensitive individuals by the RAST. The results with each of the purified allergens and WRW are shown in Tables III and IV and Fig. 9.* As expected,

*The criterion chosen for positive RAST results, i.e., greater than twice the normal control's counts bound, was more stringent than that of Berg, Bennich, and Johansson.²⁶ These authors found that, of 283 patients with positive provocation tests, 217 patients (76.7%) had RAST results greater than 1.5 times the normal control.

AgE showed a similar pattern of serum IgE antibody levels when compared to WRW. Also, almost all of the sera had elevated IgE antibody levels with AgK. In contrast, many sera were unreactive with the basic protein allergens, Ra3, Ra4, and Ra5.

Table V further illustrates the importance of AgE among ragweed-sensitive patients and emphasizes the lack of cross-reactivity among the basic protein allergens. Fifteen patients had elevated IgE antibody levels with Ra3, as well as AgE and AgK, but did not have elevated IgE antibody levels detected by solid-phase Ra4 and Ra5. Conversely, 10 patients had elevated IgE antibody levels with either or both solid-phase Ra4 and Ra5 without elevated IgE levels to Ra3. Seventeen patients had elevated IgE antibody levels with Ra4 without elevated IgE antibody levels

to solid-phase Ra5; 5 patients had elevated IgE antibody levels with Ra5 without elevated IgE antibody levels to solid-phase Ra4.

DISCUSSION

In the first set of these experiments we compared the reactivity of the purified allergens from ragweed pollen by leukocyte histamine release and by the RAST. Initially, a correlation between IgE antibody levels by the RAST, using 50 μ l of serum per RAST determination, and histamine release sensitivity was examined. This volume of serum placed the AgE and AgK RAST results for certain highly reactive sera onto the relatively nondiscriminating plateau region of the sigmoidal log-log RAST dose-response curve.^{27,28} Therefore, 5 μ l of sera were retested with solid-phase AgE and AgK. This reduced volume of sera provided AgE and AgK RAST results on the more sensitive region of the RAST dose-response curve. For the solid-phase Ra3, a serum volume of 50 μ l provided the more sensitive discrimination for all patients. As a consequence, a significant correlation was observed between the leukocyte histamine release results and the RAST for all three allergens. These correlations indicated that the allergenicity of these ragweed allergens can be evaluated by either histamine release or the RAST technology by using a representative population of ragweed-sensitive patients.

In the RAST inhibition study we found nonparallel slopes between the inhibition lines for fluid-phase AgE and AgK with solid-phase AgE and, therefore, we concluded that the fluid-phase AgK shared some allergenic determinants with AgE. The observation of extensive cross-reactivity was confirmed in the inhibition experiments with solid-phase AgK in which fluid-phase AgE and AgK inhibited in a nonparallel manner. This allergenic cross-reactivity had been observed earlier in the histamine release from sensitized leukocytes.² Fluid-phase AgE was a more potent inhibitor than AgK in both the homologous solid-phase AgE system and the solid-phase AgK system by mass and on a molar basis, because the molecular weights of these two allergens are approximately 38,000 daltons.² While these results are surprising, it is possible that IgE antibody levels to AgE are in large excess compared to IgE antibody levels to AgK in the serum pool (Table I) or that the AgK was chemically modified by the coupling procedure. The RAST inhibition study also confirms an earlier observation⁶ utilizing histamine release from sensitized leukocytes which showed the complete lack of allergenic cross-reactivity between Ra3 and AgE. In our studies, Ra3 was unable to inhibit solid-phase AgE and AgE was

TABLE IV. Summary of patient survey by RAST

Solid-phase allergen	Number of patients >2x normal in RAST	% of patients >2x normal in RAST
WRW	65/65	100%
AgE	63/65*	96.9%
AgK	57/65	87.7%
Ra3	33/65	50.8%
Ra4	23/65	28.3%
Ra5	11/65	16.9%

*The patients (110, 127) who had less than twice normal reactivity with AgE also had the lowest reactivity (<6x normal) with WRW. These same two patients showed reactivity less than twice normal with all the other purified allergens.

unable to inhibit solid-phase Ra3. The virtual lack of inhibition by the other purified allergens in the solid-phase Ra3 and Ra5 experiments indicates the allergenic purity of these preparations and reveals their lack of cross-reactivity with the other ragweed allergens. Although solid-phase Ra4 was inhibited with a 310-fold mass excess of Ra5, a 430-fold excess of Ra3, and a 460-fold excess of AgE, the small difference in RAST binding (<8-fold) between the normal control serum and the Ra4 serum pool (Table I) meant that the inhibition lines were not as statistically reliable as in the other allergen systems. The scarcity of sera with highly elevated levels of IgE antibodies for the solid-phase Ra4 used in this study could be attributed to one or both of the following: (1) the partial alteration of the allergen as a result of the CNBr coupling technique, or (2) some of the determinants on the Ra4 molecule could be sterically hindered by the coupling process to the cellulose. Either slight cross-contamination (less than 0.33% by mass) or weak cross-reactivity may explain the weak inhibition observed for all the allergens other than Ra4 with solid-phase Ra4. For example, the inhibition by Ra5 was probably due to a trace contamination by Ra4 (0.32% by mass or 0.07% on a molar basis) even though the slopes of the inhibition lines for Ra4 and Ra5 with solid-phase Ra4 were statistically different ($F_{1,11} = 7.4$, $p < 0.025$). Cross-contamination appeared probable because the Ra4 serum pool (Table I) had very low levels of IgE antibodies with solid-phase Ra5. Conversely, fluid-phase Ra4 (assuming no cross-contamination) would not be expected to inhibit the solid-phase AgE, AgK, Ra3, Ra5, or whole ragweed pollen extract because the serum pool used in these systems apparently had very low levels of IgE antibodies to Ra4 (Table I). Because the fluid-phase WRW inhibited the solid-phase Ra3 and Ra5, these allergens were present in the fluid extract. Hence, the limited inhibition of the solid-phase whole ragweed

TABLE V. Analyses of patterns of RAST reactivity with the purified allergens

Solid-phase allergen	Number of patients with RAST >2x normal to only allergens listed	
WRW	2	(3.1%)
WRW, AgE	4	(6.2%)
WRW, AgE, AgK	16	(24.6%)
WRW, AgE, AgK, Ra3	15	(23.1%)
WRW, AgE, AgK, Ra3, Ra4	11	(16.9%)
WRW, AgE, AgK, Ra3, Ra4, Ra5	5	(7.7%)
WRW, AgE, AgK, Ra3, Ra5	2	(3.1%)
WRW, AgE, Ra4	1	(1.5%)
WRW, AgE, Ra4, Ra5	1	(1.5%)
WRW, AgE, AgK, Ra4	5	(7.7%)
WRW, AgE, AgK, Ra5	3	(4.6%)
Total	65	

TABLE VI. Percentages of purified protein in ragweed pollen extracts

Purified allergen	Percent of mass of WRW as allergens	Percent of mass of pollen protein as antigens ²⁹
AgE	4.1%	6%
AgK	22.9%	3%
Ra3	0.71%	0.4%
Ra4	8.3%	0.9%
Ra5	0.44%	0.1%

pollen (prepared from the same WRW extract) by Ra3 and Ra5 might be due to: (1) Ra3 and Ra5 comprising only a very small proportion of the coupled materials, (2) steric blocking of the solid-phase Ra3 and Ra5 by the other allergens bound to antibodies, or (3) the serum pool probably has higher levels of IgE antibody to AgE compared to IgE antibody levels to Ra3 or Ra5 (Table I).

With the exception of the solid-phase AgE, all of the allergens provided inhibition lines with the homologous fluid-phase allergen and with the WRW extract that had slopes that did not differ statistically. Because comparable slopes are taken to indicate qualitative allergenic similarity,²² the RAST inhibition technique utilizing the purified allergens as the solid-phase materials provides a ready and sensitive technique to assay crude materials for both qualitative and quantitative allergen content. The sensitivity of this method was especially evident in the example of solid-phase Ra5 where 50% inhibition was achieved with 6.4 ng (1.2 pmoles) of fluid-phase Ra5 per tube. The WRW extract used to inhibit the solid-phase Ra5 required 1,450 ng per tube for 50% inhibition, indi-

cating a mass percentage of 0.44% of Ra5 in the lyophilized extract. Table VI lists the results for all allergens for comparison with values published earlier for the percentage of antigen protein compared to total protein. As indicated above, the AgE in the WRW extract showed a qualitative difference from the purified AgE on the basis of nonparallel inhibition slopes; hence, the percentage result in Table VI for AgE quantitates some but not all of the possible AgE determinants. The nonparallel inhibition curves between fluid-phase AgE and WRW extract with the solid-phase AgE (Fig. 3) may be due to a loss of allergenic sites due to polymerization of the AgE in the WRW preparation as a result of lyophilization.* A loss of antigenicity for purified AgE after lyophilization was observed earlier,³¹ but loss of antigenicity for AgE in crude ragweed extracts after lyophilization was found to be less than 20%.³² Alternatively, the nonparallel inhibition may be due to the AgK or some other cross-reactive allergen occurring in the WRW extract. As shown in Table VI for solid-phase AgK, a large percentage of the WRW extract, almost 23% by mass, possesses determinants qualitatively similar to AgK. A portion of this reactivity must be attributed to the AgE in the WRW extract because: (1) the purified AgE was a more potent inhibitor than AgK with solid-phase AgK and (2) cross-reactivity between AgE and AgK has been well documented.²

Previous publications concerned with the purified ragweed allergens have studied selected pairs of the allergens: AgE and AgK,² AgE and Ra3,⁶ and AgE and Ra5.³³ Our study investigated the reactivity of all

*The purified AgE used throughout this study had never been lyophilized at any stage in its purification,³⁰ but the WRW was lyophilized (see section on Materials and Methods).

five allergens on a 65-patient population. We found AgE to be the dominant allergen, i.e., no other solid-phase allergen showed RAST reactivity with a patient's serum unless that patient also reacted with solid-phase AgE. The earlier study comparing AgE and AgK² found that by direct skin testing with AgE and AgK 37 patients had positive tests with both allergens and by leukocyte histamine release 21 patients had positive tests with both allergens. Our study using the RAST showed that of the 63 patients with IgE antibodies to AgE, 57 had IgE antibodies to AgK. Although we detected no cross-reactivity between AgE and Ra3, IgE antibodies reactive with Ra3 were found in those 33 patients who also reacted with both AgE and AgK. The earlier study comparing AgE and Ra3⁶ showed 16 of 22 patients releasing over 50% histamine with both AgE and Ra3 by leukocyte histamine release and 12 of 19 patients giving positive direct skin tests with both allergens. Patients with IgE antibodies reactive with Ra4 or Ra5 showed a variety of patterns of reactivity with each other and with AgK and Ra3. Previously, direct skin testing with Ra4 on a different group of ragweed-sensitive patients showed 6 of 63 with reactions of 2+ and 2 of 63 with reactions of 1+.³⁴ Utilizing the RAST, we found elevated levels of IgE antibodies to Ra4 in 23 of 65 ragweed-sensitive patients. Sera with IgE antibodies reactive with both Ra5 and AgE (11 of 63 or 17%) compare well with earlier results³³ by direct skin testing where 18 of 105 or 17% had strongly positive results with both AgE and Ra5. These data provide additional evidence for the concept of individual patterns of patient reactivity to the multiple allergens derived from a common source, which was previously observed with the rye grass pollen allergens.³⁵⁻³⁷

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