

In vitro cross-allergenicity of major aeroallergenic pollens by the radioallergosorbent technique

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Cross-reactivity between major classes of aeroallergenic pollens has been estimated by comparing the inhibitory effects of selected allergens upon the reaginic activity of other native and non-native varieties, as measured by the radioallergosorbent technique (RAST). Common allergenic determinants were demonstrated in indigenous and nonindigenous ragweed species. Although patterns of inhibitory capacity were less uniform among grass pollens, endemic species tended to have more common allergenic properties than nonendemic species. Tree pollens exhibited the greatest degree of heterogeneity, confirming the previous view that these pollens tend to be less cross-reactive. Concomitant skin test threshold studies utilizing the same groups of pollens were in general agreement with in vitro results. Because threshold skin testing was affected by a number of uncontrollable variables, however, the current modification of RAST in estimating cross-allergenicity was more accurate and reliable than data based upon cutaneous tests. It was concluded that reagin neutralization by the RAST method is the best currently available method of assessing cross-allergenic properties of pollens.

Species cross-reactivity within major classes of aeroallergens such as ragweeds, grasses, and trees has been recognized for many years. Extensive experience with direct cutaneous tests has demonstrated a high percentage of simultaneous positive reactions to various species of ragweed. In addition, the occurrence of positive tests to foreign species of composites (e.g., the South American weed, tithona) in ragweed-sensitive patients, tends to confirm the presence of cross-reactions within this botanical family.² Variations in intensity of reactions to different species of grass pollens in the same patient have also suggested cross-reactivity among these aeroallergens.³ The fact that grass-sensitive patients may exhibit positive skin tests to nonindigenous species lends further credence to

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Supported in part by National Institutes of Health Training Grant No. AM 05509-08.

Presented in part at the Annual Meeting of the American Academy of Allergy, Jan. 19-23, 1974, Miami Beach, Fla.

Received for publication Oct. 23, 1974.

Accepted for publication Dec. 17, 1974.

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TABLE I. List of pollen extracts used for RAST and skin test titrations

Composites	Grasses	Trees
*Short ragweed (SR) (<i>Ambrosia elatior</i>)	*Timothy (T) (<i>Phleum pratense</i>)	*Box elder (BE) (<i>Acer negundo</i>)
*Giant ragweed (GR) (<i>Ambrosia trifida</i>)	June (J) (<i>Poa pratensis</i>)	*Elm (E) (<i>Ulmus americana</i>)
*False ragweed (FR) (<i>Franseria acanthicarpa</i>)	Orchard (OR) (<i>Dactylis glomerata</i>)	*Oak (O) (<i>Quercus alba</i>)
*Western ragweed (WR) (<i>Ambrosia psilostachya</i> Gray)	Sweet vernal (SV) (<i>Anthoxanthum odoratum</i>)	Beech (B) (<i>Fagus grandifolia</i>)
	*Meadow fescue (MF) (<i>Festuca elatior</i>)	Hazel nut (HN) (<i>Corylus avellana</i>)
	*Rye (R) (<i>Lolium perenne</i>)	Gray alder (GA) (<i>Alnus incana</i>)
	*Bermuda grass (BG) (<i>Cynodon dactylon</i>)	Birch (BI) (<i>Betula verucosa</i>)
	Common reed (CR) (<i>Phragmites communis</i>)	Mountain cedar (MC) (<i>Juniperus sapinoides</i>)

*These pollen extracts were also used as inhibitory reagents in cross-inhibition studies.

the existence of cross-reactivity in various members of the grass family.⁴ In the course of past studies it was observed that certain species such as Bermuda grass possessed unique allergenic determinants which could be differentiated from the majority of grass pollens.⁵ Skin test surveys also indicated that different genera of tree pollens exhibited less cross-reactivity than grass or ragweed pollens.⁶ All skin test data purporting to measure cross-allergenicity, however, are subject to the valid criticism that specific activity of various allergens can be compared only in patients having equivalent degrees of skin sensitivity, a principle frequently ignored or overlooked.⁷ This objection does not pertain to the procedure which measures the neutralization of passively transferable reagin,⁸ but the risks of serum hepatitis inherent in this method have limited its wider application.

In recent years immunochemical methods have been employed to fractionate various allergenic extracts.⁹⁻¹¹ Although these derivatives have permitted more effective in vitro investigation of cross-immunogenicity by a number of methods,¹²⁻¹⁴ this type of evidence is circumstantial and the potential role of these products as cross-allergenic reactants is ultimately dependent upon bioassays of passively sensitized skin,¹⁵ leukocytes,¹⁶ and chopped human lung.¹⁷

When in vitro determination of allergen potency was made possible by several simple modifications of the radioallergosorbent technique (RAST),^{18, 19} It seemed possible that one of these modifications, the inhibitory capacity of allergenic extracts,²⁰ could be utilized to evaluate cross-allergenicity. In the current investigations we have accomplished this goal in the major groups of aeroallergenic pollens.

METHODS AND MATERIALS

Patients

Five hundred untreated allergic patients were screened for the presence of specific IgE to a battery of pollen allergens (Table I) by the RAST technique. Patients demonstrating high RAST scores (3+ or >) were selected for a multiple series of scratch test titrations

TABLE II. Classes of sera

Retained radioactivity (cpm for unknown serum)	Retained radioactivity (cpm for reference serum diluted)	RAST class
Same or >	1:1	4
Between	1:1-1:5	3
Between	1:5-1:25	2
Between	1:25-1:50	1
Same or <	1:50	0

performed with the same allergens* ranging from 2×10^{-1} to 10^{-6} g/ml. The end-point threshold of these titrations was recorded as the highest dilution resulting in a 2+ skin test.† Scratch tests were chosen because previous experience in our laboratory had demonstrated that their correlation with RAST was much better than intracutaneous tests.

Serological: RAST

The RAST was performed as previously described, with minor modifications. Each allergen was coupled to methylcellulose discs previously activated by cyanogen bromide.‡ These discs were incubated with the serum sample for 3 hr and, after repeated washing, with ^{125}I -radiolabeled anti-IgE§ for 16 hr. After another washing, residual radioactivity of disc reactants was counted in a gamma counter. All experiments were conducted in duplicate. Mean radioactivity counts were compared to counts of reference allergen-coupled discs‡ incubated with serial dilutions of a highly reactive reference serum.‡ The results were expressed as classes from 0-4 as shown in Table II. Classes of 2 or greater were considered positive; a class of 1 was interpreted as equivocal. All sera were tested prior to the respective pollen seasons.

Allergen inhibition

Cross-inhibitory studies were performed with sera showing high titers of RAST reactivity to ragweed, grass, and tree allergens in the preliminary survey. This selection process did not necessarily include all patients showing multiple skin test threshold responses. The degree of RAST reactivity was defined as a minimum binding capacity of at least 60% (absolute radioactivity, approximately 3,000 cpm) as compared to the reference serum (absolute radioactivity, approximately 5,000 cpm). In the ragweed experiments 5 individual sera and 4 allergen absorbents (false ragweed, western ragweed, short ragweed, and giant ragweed) were tested (Table I). Four grass species (meadow fescue, timothy, rye, and Bermuda) representing 3 tribes were selected as grass absorbents. Five sera were available for experiments with meadow fescue, timothy, and rye, and 3 sera were suitable for absorption with Bermuda grass.§ Elm, oak, and box elder were utilized as representative agents in assaying cross-allergenicity among tree allergens. Five sera were considered to have sufficient RAST activity and inhibitory capacity for elm and oak experiments; 4 sera were available for absorption with box elder.

Equal volumes (1 ml) of these reference sera and 3 dilutions (1:10, 1:100, and 1:1000) of various allergen absorbents were incubated at 37° C for 2 hr and then centrifuged at 1,500 $\times$ g for 15 min. The upper two thirds of this reaction mixture was carefully removed and

*Purchased from Greer Laboratories, Lenoir, N. C.

†A wheal diameter twice the size of the control test site.

‡Supplied by Pharmacia, Ltd., Piscataway, N. J.

§High-potency reaginic sera to Bermuda grass were more difficult to find, presumably because this allergen is not endemic to Cincinnati. Two grass sera were rejected as suitable reagents because their activities were inhibited by nonendemic allergens (Bermuda grass, common reed) slightly more at lower concentrations than at higher concentrations of allergens. The reason for this paradoxical effect was not determined.

TABLE III. Incidence of positive RAST within major pollen groups

	%
<i>Composites* (94)†:</i>	
SR	96
GR	73
FR	96
WR	88
<i>Grasses* (50):</i>	
T	96
J	86
OR	72
SV	88
MF	90
R	86
BG	63
CR	48
<i>Trees* (35):</i>	
BE	77
E	67
O	63
B	66
HN	60
GA	46
BI	32
MC	14

*Refer to Table I for definition of abbreviations.

†Numbers in parentheses denote the number of sera tested to various species within each major pollen group.

incubated with a series of activated methylcellulose discs coupled with the various allergens of each particular group (Table I).^{*} The RAST was then determined according to the procedure described above. The degree of inhibition of RAST reactivity was obtained by comparing residual radioactivity of the absorbed samples to that of a control sample containing reference serum alone but diluted to comparable volume with normal saline. Mean values of individual experiments were plotted on semilogarithmic scales, from which results could be expressed as the dilution of absorbing allergen causing 50% inhibition of RAST activity in ragweed and grass experiments or 33% inhibition of RAST activity in tree experiments

RESULTS

Frequency profiles of RAST screening

Ninety-four sera contained high RAST titers to one or more of the 4 ragweed species tested (Table III). Most of these samples (96%) exhibited positive reactivity to short ragweed (SR) and false ragweed (FR), but significant percentages of positive RAST scores were also obtained with western ragweed (WR) and giant ragweed (GR). Isolated specific reactivity was demonstrated to FR in a single instance and to SR in 3 cases. Thus, as predicted, ragweed reaginic sera demonstrated almost uniform reactions to all 4 ragweed allergens.

Fifty serum samples showed RAST reactivity to 1 or more of 8 species of grasses (Table III). The percentage of positive reactions to timothy (T), meadow fescue (MF), sweet vernal (SV), rye (R), and June (J) grasses was approximately equal (86% to 96%) as contrasted with Bermuda grass (BM) and common reed (CR), both of which had lower percentages of 63% and 48%, respectively.

^{*}Supplied by Pharmacia, Ltd., Piscataway, N. J.

TABLE IV. Inhibitory capacities of representative species of the major pollen groups

	Composites*: Reciprocal of absorbent dilution causing 50% inhibition			
	FR (5)†	WR (5)	SR (5)	GR (5)
FR	>10,000	4,500	1,500	150
WR	1,300	225	130	45
SR	850	125	125	25
GR	350	200	70	80

	Grasses*: Reciprocal of absorbent dilution causing 50% inhibition			
	MF (5)	T (5)	BG‡ (3)	R (5)
MF	8,000	1,000	<10	2,000
T	100	4,500	<10	40
BG	NI§	NI	30	<10
R	400	1,700	NI	1,000
J	25	1,200	NI	30
OR	50	150	NI	250
SV	225	1,200	NI	250
CR	NI	NI	<10	NI

	Trees*: Reciprocal of absorbent dilution causing 33% inhibition		
	BE (4)	E (5)	O (5)
BE	18	120	32
E	<10	220	9
O	<10	99	290
B	<10	190	50
NH	NI	120	40
GA	NI	64	10
BI	NI	180	40
MC	NI	NI	NI

*See Table I for definitions of abbreviations.

†Numbers in parentheses refer to the number of sera tested for each absorbent.

‡Maximum inhibitory capacity of BG was 40%.

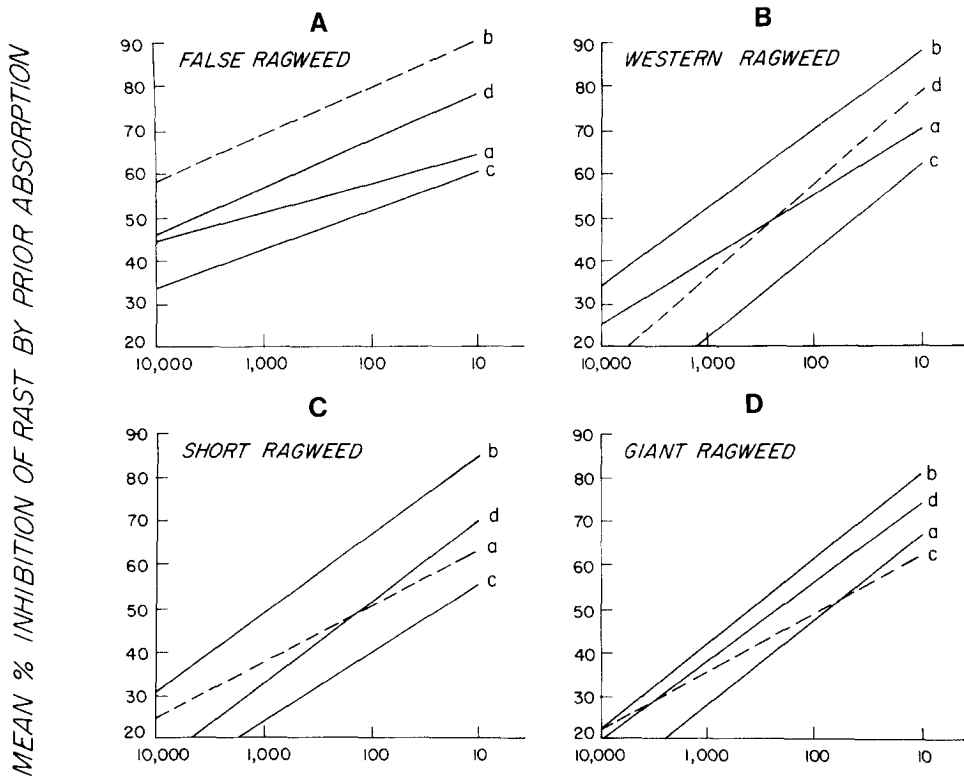
§NI: No inhibitory activity demonstrated at the 50% level for ragweed and grasses or at the 33% level for trees.

It is evident that results in the nonindigenous species reduced the over-all uniformity of response among the grass pollens.

Table III also lists the frequency pattern of 8 tree pollens tested in 35 sera which had shown RAST reactivity to 1 or more of these allergens. This profile may be characterized by a general lack of uniformity with a higher percentage of positive reactions among prevalent pollens (box elder [BE], elm [E], beech [B], and oak [O]), intermediate results among secondary flora (hazel nut [HN], gray alder [GA], and birch [BI]) and a decreased incidence of the nonprevalent species, mountain cedar (MC). In fact, the rank order among the tree allergens obtained during this RAST survey coincides remarkably well with ecology of tree flora in the Cincinnati area.

Cross-inhibition experiments

Significant inhibition of RAST activity to all 4 ragweed species was observed when each of the 4 pollen extracts was used as the absorbing allergen (Table IV, Fig. 1). Further, there was a linear relationship between the degree of inhibition and concentration of the absorbent. Not only was FR the best homologous absorbent (Table IV, Fig. 1, A) but also its antibodies were most



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FIG. 1. Measurement of cross-allergenicity among composite aeropollens by RAST inhibition. (A) False ragweed, (B) western ragweed, (C) short ragweed, and (D) giant ragweed pollen extracts were used as homologous allergen absorbents before determining RAST activity of 5 ragweed reference sera with (a) short ragweed (SR), (b) false ragweed (FR), (c) giant ragweed (GR), and (d) western ragweed (WR) allergen discs. Inhibition lines were drawn after mean inhibitory percentages of the 5 reference sera were plotted at 3 dilutions of homologous absorbent (1:10, 1:100, and 1:1,000).

effectively absorbed by the other ragweed species. Paradoxically, homologous absorption by short ragweed was less effective than by the nonindigenous species of false and western ragweeds.

The slopes of these inhibition curves revealed some interesting relationships (Fig. 1). Except for false ragweed, homologous inhibition curves intersected at least 1 or several of the heterologous curves, indicating the presence of non-cross-reacting determinants in the homologous allergen. In all experiments the slopes of FR and WR were parallel. Projected vectors of the inhibiting curves of SR and GR were always convergent, however, and in one instance there was actual transection of these 2 slopes (Fig. 1, D).

Predictably, the cross-absorption patterns of grass allergens were more complex (Table IV, Fig. 2). The inhibitory function of homologous allergens exceeded 50% except for BG, which absorbed only slightly more than 40% of its

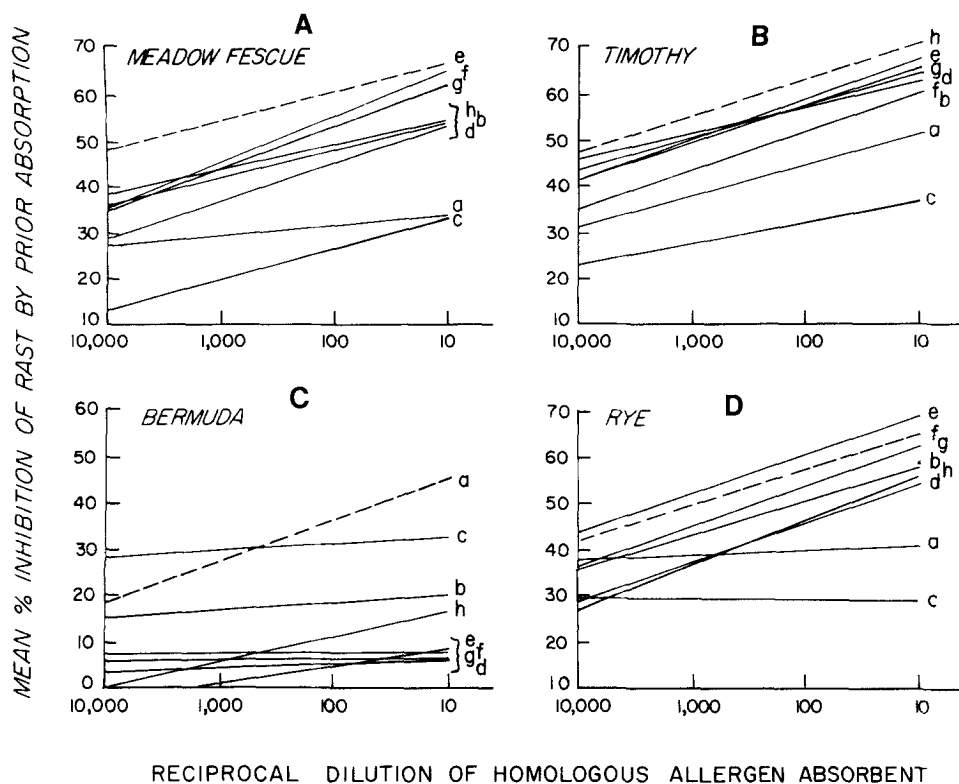


FIG. 2. Measurement of cross-allergenicity among grass aeropollens by RAST inhibition. (A) Meadow fescue, (B) timothy, (C) Bermuda, and (D) rye pollen extracts were used as homologous allergen absorbents before determining RAST activity of reference sera with (a) Bermuda (BG), (b) orchard (OR), (c) common reed (CR), (d) June (J), (e) meadow fescue (MF), (f) rye (R), (g) sweet vernal (SV), and (h) timothy (T) allergen discs. Five reference sera were used in experiments A, B, D, and 3 reference sera were employed in experiment C. Inhibition lines were drawn after mean inhibition percentages of the reference sera were plotted at 3 dilutions of homologous absorbent (1:10, 1:100, and 1:1,000).

homologous antibody. Similar to ragweed experiments, there was a linear relationship between inhibitory capacity and concentration of immunoabsorbent. On the basis of both general appearance and degree of inhibition, it is evident that grass inhibitory curves may be divided into two major categories. The indigenous species (T, OR, J, SV, MF) show similar absorption profiles, both quantitatively (Table IV) and qualitatively (Fig. 2), whereas nonprevalent pollens (BG, CR) dissociate markedly in all experiments (Table IV, Fig. 2). These dissimilarities are clearly shown in Fig. 2, C, when BG was employed as the homologous absorbent. This experiment also indicates that there is a distinct allergenic difference between BG and CR. Because R contains some antigenic determinants not thought to be present in T, absorption experiments involving these allergens were analyzed in greater detail (Fig. 2, B and D). In the T experiment, for example, the slopes of MF, SV, and OR were almost parallel and the slopes

TABLE V. Mean reciprocals of scratch test end-point thresholds of major aeroallergenic pollens

	Composites (4)*		Grasses (6)*		Trees (5)*	
Endemic	SR	540	T	210	BE	50.6
	GR	2,760	SV	73	E	
					O	
					B	
Nonendemic	WR	305	R	60	HN	150
	FR	100	MF	87	GA	
					BI	

*Numbers in parentheses indicate the number of patients tested. See Table I for abbreviations of endemic and nonendemic species.

of JG and R diverged slightly from that of the homologous absorbent. If the same comparisons are made in the R experiment (Fig. 2, *D*), a much closer degree of agreement is observed among the indigenous grass species with the exception of T. These absorption experiments, therefore, suggested the presence of minor allergenic differences among the indigenous grasses and major allergenic differences between endemic and nonendemic varieties.

Cross-absorption experiments of tree pollens (Table IV, Fig. 3) illustrated important differences from those performed with either ragweed or grass pollens. It was clearly demonstrated that the tree pollen extracts used in these experiments were poorer immunoabsorbents than ragweed or grass allergens. Thus, the indigenous tree pollens (O, BE; Fig. 3, *A* and *C*) absorbed slightly more than 33% activity from their corresponding antibodies. Although the inhibitory capacity of E was somewhat better (approximately 60%), it did not reach levels comparable to those observed in ragweed and grass experiments (80% to 90%). In all tree cross-inhibition experiments there appeared to be less concordance between inhibition curves of the respective homologous and heterologous allergens than was previously observed in ragweed and grass experiments. It was also noteworthy that a complete lack of allergenic association between a nonindigenous tree species, MC, and other tree pollens was obtained in all experiments. In the BE cross-absorption studies (Fig. 3, *A*) inhibitory curves of species of secondary importance in the Cincinnati area (HN, G, GA) differed significantly from similar data for the homologous allergen (BE) and other tree allergens (E, O, B) endemic to this locale.

Skin test titration

Since patients were selected for skin threshold testing on the basis of high RAST scores, all patients exhibited positive scratch tests (at the 1:20 dilution) to one or more of the allergens surveyed in each major pollen group. Results of end-point titrations were variable, however. This phase of the investigation was undertaken to estimate skin cross-reactivity by differences of end-point threshold responses between indigenous and nonindigenous species within each class of pollen. Very often, however, an individual patient would demonstrate satisfactory threshold reactions to endemic species but not to nonendemic varieties. In grass-sensitive patients, positive titrations for Bermuda grass were

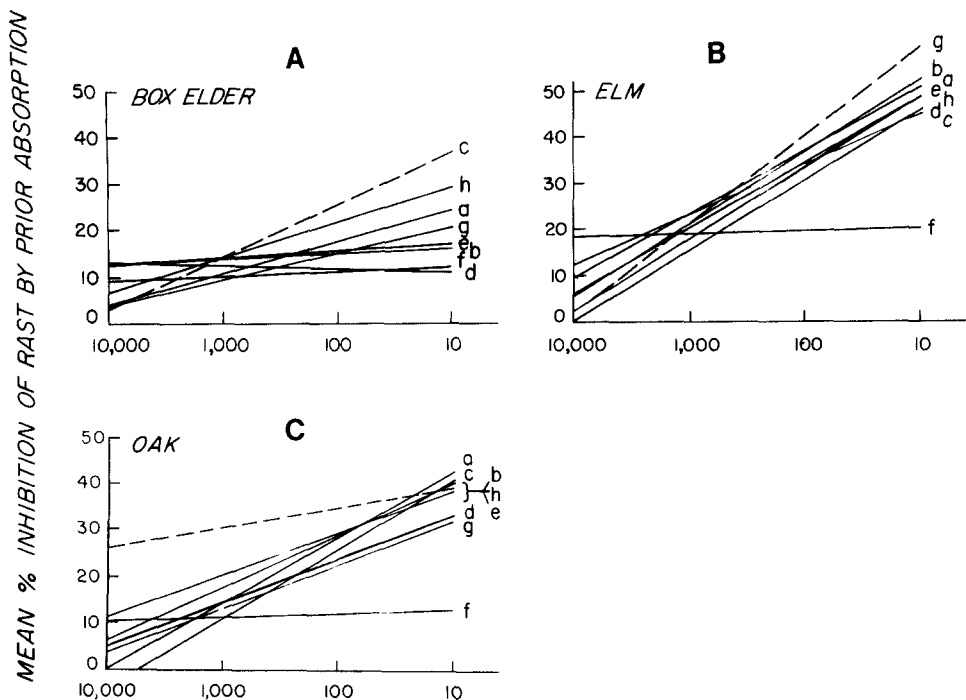
the exception rather than the rule and threshold responses to mountain cedar could not be shown in any of the tree-sensitive patients. Analysis of these data was therefore limited to specific patients whose titration results encompassed both indigenous and nonindigenous species within each major pollen group (Table V). It was possible to compare all 4 ragweed species in 4 patients in whom direct skin test threshold titers of endemic pollens (SR, GR) were clearly higher than nonendemic ones (WR, FR). Four grass pollen varieties were compared in 6 grass-sensitive patients. The highest threshold response was observed to T, the predominant grass pollen in Cincinnati, whereas threshold titers were at least twofold lower to grass pollens (SV, R, MF) of secondary importance in this area. Direct threshold skin testing in tree-sensitive patients exhibited the widest variability so that comparable data could be obtained only by pooling 4 indigenous (BE, E, O, B) and 3 nonindigenous tree pollens (HN, GA, BI) in 5 of these patients. This analysis unexpectedly revealed a higher threshold titer to nonendemic than to endemic species.

DISCUSSION

Although considerable information about cross-allergenicity of atopic allergens has been gleaned from direct cutaneous testing and *in vivo* reagin neutralization, the limitation of accuracy and reproducibility of the former and the hazards of the latter are well established. In the current investigation skin test end-point thresholds were employed to compare specific activities of various pollen allergens in the hope that semiquantitative differences between homologous and heterologous allergens would indicate the presence of cross-allergenic components. Although these experiments generally suggested the presence of cross-allergenicity between various species of grass and ragweed, results in tree-sensitive patients varied so widely that meaningful interpretation was impossible. In addition, the number of patients exhibiting threshold responses to all the allergens we wished to compare was so few that over-all results of these skin tests would hardly be considered conclusive. Moreover, variations in threshold response could simply indicate the existence of absolute differences of skin sensitivity to indigenous and secondary allergens in individual patients.

In contrast to skin test studies, cross-allergenicity and allergenic differences among the major pollen groups were both clearly demonstrated *in vitro* by the RAST. Simple RAST screening by a battery of ragweed, grass, and tree pollens suggested the presence of cross-reactive determinants among ragweed and grass species and the relative differences in allergenic reactivity that have been formerly recognized among tree pollens.

Perhaps the ragweed experiments best illustrate the potential usefulness of RAST in determining cross-allergenicity. The majority of ragweed reaginic sera exhibited positive reactions to WR and FR, species not endemic to southwestern Ohio. Since most patients in the test population have been life-long residents of this locality, natural sensitization to these foreign species could not have occurred. Cross-absorption studies further confirm this assumption because absorption with any one of the ragweed species would significantly inhibit the RAST titers of the heterologous allergens. The inhibiting characteristics of



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FIG. 3. Measurement of cross-allergenicity among tree aeropollens by RAST inhibition. (A) Box elder, (B) elm, and (C) oak pollen extracts were used as homologous allergen absorbents before determining RAST activity of reference sera with (a) beech (B), (b) birch (Bl), (c) box elder (BE), (d) gray alder (GA), (e) hazel nut (HN), (f) mountain cedar (MC), (g) elm (E), and (h) oak (O) allergen discs. Four reference sera were used in experiment A and 5 reference sera were employed in experiments B and C. Inhibition lines were drawn after mean inhibitory percentages of the reference sera were plotted at 3 dilutions of homologous absorbent (1:10, 1:100, and 1:1,000).

FR are somewhat paradoxical, however. At the 50% inhibitory level, this allergen has the most potent inhibitory capacity and its reagent is also more easily inhibited by the other ragweed pollen absorbents. These observations would be explicable if FR contained larger or more extractable amounts of the sensitizing allergen(s). Unpublished experiments have, in fact, demonstrated that concentrations of antigen E (prepared from SR) in FR are higher than in SR.* Whether this active allergenic component is responsible for the unusual response of FR in the current assay is a subject for future research.

The RAST technique was also effective in comparative analysis of the allergenic properties of grass pollens. Clear-cut differences between endemic (T, J, OR, SV, MF) and nonendemic species (BG, CR) were recognized in both

*This laboratory has confirmed this by gel diffusion experiments in which antigen E content of various ragweed species was estimated by reaction of various ragweed extracts with sheep anti-antigen E serum.

screening and cross-inhibition experiments. Inhibitory capacity of rye pollen was of special interest inasmuch as several purified fractions (rye group antigens I, II, and III) have been isolated from this allergen.¹⁰ When R was employed as the immunoabsorbent (Fig. 3, *D*), inhibitory curves of all grass pollens, save the nonindigenous varieties (BG, CR), pursued almost parallel courses to the homologous absorbent. It might be postulated that timothy was a "rye group pollen" in contrast to a previous report that this pollen demonstrated only weak cross-immunogenicity with rye group I antigens.²¹ Subsequently this assumption was shown to be premature since R inhibitory curves in both MF and T experiments (Fig. 3, *A* and *B*) assumed divergent paths from the respective homologous absorbent curves. It was also noteworthy that the inhibitory capacity of MF exceeded homologous inhibition by R (Fig. 3, *D*). This could be analogous to the behavior of false ragweed in the composite experiments inasmuch as there is evidence that MF contains a slightly greater concentration of rye group I antigen than R.²¹ The RAST appears to be an ideal method of testing this particular allergenic relationship, particularly if the *in vitro* inhibitory capacity of rye group I, II, and III antigens could be determined directly.

RAST survey results and cross-absorption patterns revealed more heterogeneity among endemic and nonendemic tree pollens, thereby confirming the clinical aphorism that these allergens are much less likely to cross-react. In addition, these investigations conclusively demonstrated almost complete lack of allergenic identity between a nonendemic species (MC) and endemic varieties. Further, BE inhibition studies clearly showed different allergenic determinants between the homologous allergen and secondary plants such as HN, GA, and BI.

These investigations seem to indicate that the RAST fills a long-standing void as a valid and simple *in vitro* assay of cross-allergenicity. Because it measures specific allergenic activity directly and quantitatively, circuitous interpolations about the possible interrelationships of immunogenic and allergenic determinants are irrelevant. It may be performed more rapidly and less expensively than the techniques of passive leukocyte or chopped lung histamine release. More important, however, it has none of the biologic disadvantages of cutaneous testing and it completely obviates risk factors entailed in *in vitro* reagin neutralization methods. We believe that this technique should be the method of choice in assessing cross-allergenic properties of crude extracts as well as allergenic fractions of pollens and other atopic allergens.

We wish to express our appreciation for the excellent technical efforts of Ms. Mary Earley and Mr. George Peters.

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