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Allergenic Cross-Reactivity between *Callistemon citrinis* and *Melaleuca quinquenervia* Pollens

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Abstract. Aqueous extracts of both *Callistemon citrinis* (bottlebrush) and *Melaleuca quinquenervia* (melaleuca) were analyzed for allergenic cross-reactivity. Inhibition analysis using the radioallergosorbent test (RAST) was performed on the ammonium bicarbonate extracts of bottlebrush (NH_4B) and melaleuca (NH_4M) pollens. RAST inhibition analysis demonstrated that the extracts contained allergenically cross-reactive components. Sephadex G-100 column chromatography of NH_4B and NH_4M extracts resulted in at least 4 distinct peaks for each extract analyzed. These fractions were designated NH_4B1–NH_4B4 and NH_4M1–NH_4M4. A modified dot-blot assay for detection of allergenic components was utilized to show that the first elution peaks of bottlebrush and melaleuca, NH_4B1 and NH_4M1, respectively, contained allergenic components. These allergenic components, NH_4B1 and NH_4M1, had estimated molecular weights of 50,000 and 35,000 daltons, respectively.

Introduction

Allergenic cross-reactivity among pollen allergens has been well documented [1–5] and is frequently found among related species of trees and grasses [6]. For many years, allergists have speculated that the pollens of *Callistemon citrinis* (bottlebrush) and *Melaleuca quinquenervia* (melaleuca) might have very similar or cross-reactive allergenic components. This speculation was based upon the clinical observation that most patients who were skin test reactive to melaleuca pollen were also found to be reactive to bottlebrush pollen. A very high degree of cross-reactivity between the allergenic components of these two pollens would provide an explanation for these clinical observations and might be expected because these two trees are in the same family, Myrtaceae. These trees were imported from Australia and presently are widely distributed in Florida.

Crossed immunoelectrophoresis has previously been used to detect the antigenic components of these two pollen extracts [7]. Tandem crossed immunoelectrophoresis (TCIE) confirmed that the ammonium bicarbonate extracts of bottlebrush (NH_4B) and melaleuca (NH_4M) pollens contained antigenically cross-reactive components [7]. Trans-blot enzyme-linked crossed immunoelectrophoresis (TECIE) detected at least three and two allergenic components in the NH_4B and NH_4M extracts, respectively. These same precipitin bands were antigenically cross-reactive since they precipitated in the intermediate gel containing antisera to the cross-reactive pollen when analyzed by TCIE [7].

Cross-reactivity between allergens can also be demonstrated using the radioallergosorbent test (RAST) inhibition assays [6]. RAST inhibition studies have previously indicated that clustering of hypersensitivities to plant material exists due to the presence of IgE antibodies which cross-react with several antigens [2]. The research reported here further substantiates, by RAST inhibition analysis, the allergenic cross-reactivity between the NH_4B and NH_4M extracts of these pollens and further characterizes the allergenic components utilizing gel filtration.

Materials and Methods

Allergen Extracts

Pollens from melaleuca and bottlebrush trees were obtained by removing and collecting the anther portion of the whole flower.

Five grams of pollen were dried, ground into a very fine powder in a Wiley Mill grinder (50 mesh), defatted in a Soxhlet extractor with 200 ml diethyl ether and air-dried for 24 h. This dried material was extracted with 200 ml 0.005 *M* ammonium bicarbonate at pH 7.8 for 48 h at 4 °C since previous studies using RAST analysis demonstrated that this solvent extract bound the greatest amount of IgE from allergic patient sera [7]. The ammonium bicarbonate extracts of both pollens were centrifuged at 40,000 *g* for 20 min, filtered using 0.65-, 0.45-, and 0.22-μm HA Millipore filters and were lyophilized for storage at -20 °C.

Rabbit Antisera and Human Serum

Antisera toward bottlebrush and melaleuca pollens were prepared in rabbits as described previously [7]. Human serum samples were collected from patients who were designated as allergic or nonallergic based upon clinical testing. Patients were considered to be allergic if skin test results using bottlebrush and melaleuca pollen extracts were 2+ or greater and if they had symptoms when the respective plants were flowering [8]. Patients were considered nonallergic if skin test results were 0. The concentration of each extract used for skin testing was a 10^{-3} dilution (0.1% w/v).

RAST and RAST Inhibition

RAST inhibition was performed using the method of Gleich et al. [9] with the following modifications: Both NH_4B and NH_4M allergen extracts were bound to Sepharose-4B before incubation with allergic sera [3]. Serial 2-fold dilutions of Sepharose-Melaleuca (Sepharose-MEL) and Sepharose-Bottlebrush (Sepharose-BOT), and Sepharose-human serum albumin (Sepharose-HSA) were made, using 1 ml of packed Sepharose-antigen (Sepharose-AG) in 1 ml of RAST diluent (1:2-1:32). After centrifugation at 700 *g* for 10 min the diluent was removed from the Sepharose-AG mixtures and 1 ml of a 1-2 dilution of pooled allergic sera was added to each dilution of Sepharose-AG. Pooled allergic sera consisted of sera obtained from approximately 8 patients being skin test positive to the allergens [7]. The tubes containing the Sepharose-AG and human serum mixtures were rotated, incubated at 4 °C for 24 h and centrifuged at 5,000 *g* for 5 min. The sera were collected and RAST analysis was performed [7, 10]. RAST discs were prepared as previously described [7] for both NH_4M and NH_4B. The concentration of extracts used originally for coupling to cyanogen-bromide-activated Whatman 44 filter paper discs was varied to determine the maximum specific binding for both extracts. The concentration which produced maximal binding was approximately 5 mg/25 ml. Activated discs were incubated for 3 h with 100 μl of either uninhibited or inhibited allergic patient sera or with a normal control serum containing no IgE antibodies to the allergens. The discs were washed and incubated overnight in 50 μl of ^{125}I-labeled goat antihuman IgE. The discs were counted in a Picker (Spectroscaler 4R) counter and the results expressed as counts per minute (cpm). The percent inhibition of IgE-binding in RAST analysis was calculated according to the following formula:

$$\text{inhibition, \%} = \frac{\text{cpm (0)} - \text{cpm (x)}}{\text{cpm (0)} - \text{cpm (n)}} \times 100,$$

where cpm (0) = the cpm obtained with uninhibited sera; cpm (n) = the radioactivity bound to the discs incubated with a normal control serum containing no IgE antibodies to the allergens and cpm (x) = cpm obtained with inhibited sera [3]. Phadebas RAST unit calculations were calculated (Pharmacia Diagnostics, Uppsala, Sweden).

Sephadex G-100 Chromatography

Gel filtration was performed at room temperature using Sephadex G-100 equilibrated with 0.025 *M* Tris buffer pH 7.5 and packed into a glass column (2.5 × 90 cm). The void volume was determined using blue dextran. Twenty milligrams of either the NH_4B or NH_4M lyophilized extract were dissolved in 2 ml of Tris buffer and applied to the column. Eighty 3-ml fractions were collected at a flow rate of 10 ml/h and the eluent monitored at 277 nm. Approximate molecular weights were determined using the following standards: bovine γ-globulin (158,000 daltons), bovine serum albumin (67,000 daltons), myoglobin (17,000 daltons) and vitamin B_{12} (1,350 daltons). Molecular weights were estimated by comparing retention times of respective antigens to those of the standards.

Protein Blotting

Immunoblotting was performed as described by Bio-Rad [11] with the following modifications: rabbit antisera to melaleuca or bottlebrush pollens were used in the first antibody solution and goat antirabbit IgG horseradish peroxidase conjugate was used in the second antibody solution in place of a goat antimouse conjugate. A modified immunoblotting technique using allergic patient sera and sheep antihuman IgE horseradish peroxidase conjugate was also developed and called Allergoblot. Allergoblot was used to detect which of the NH_4B and NH_4M pollen extract fractions obtained with Sephadex G-100 chromatography was (were) allergenic. Allergoblot analysis of gel filtration fractions was performed by blotting 1 μl of each G-100 fraction onto a nitrocellulose membrane previously lined with a 6 × 6 mm grid. Unbound sites on the membrane were blocked with 3% gelatin and trisbuffered saline (TBS) solution, pH 7.5, and incubated for 18-20 h in a 1:4 dilution of allergic patient sera in 1% gelatin and TBS. Two 10-min washes using 100 ml TBS were performed on the membranes before incubating for 3 h in sheep antihuman IgE (ε-chain specific) peroxidase-labeled antibodies (United States Biochemicals) diluted 1:3,000 in 1% gelatin and TBS. Cord blood (human fetal cord serum) was obtained and used as a negative control.

Results

Competitive Inhibition Studies Using RAST Analysis

The ability of Sepharose-bound melaleuca and bottlebrush antigens to compete with NH_4B and NH_4M RAST discs for the binding of allergic patient IgE was studied using the RAST inhibition assay (table I). The percent inhibition of IgE-binding to NH_4M and NH_4N RAST discs decreased from 69-6 and 82-1%, respectively, with decreasing concentrations of Sepharose-MEL (fig. 1, 2). Similar decreases in the percent inhibition were observed for varying concentrations of Sepharose-BOT. These decreases in percent inhibition of IgE binding ranged from 84

Table I. Competitive inhibition of NH_4M or NH_4B binding to IgE by varying concentrations of Sepharose-AG

Sera		Allergen discs							
		NH_4M				NH_4B			
		cpm	score	PRU	inhibition, %	cpm	score	PRU	inhibition, %
Neg. control		438	0	0.1		606	0	0.1	
Sepharose-MEL	2	1,089	1/0	0.23	69	1,042	1/0	0.21	82
	4	1,313	1/0	0.34	58	1,461	1	0.41	65
	8	1,606	1	0.46	44	1,599	1	0.46	59
	16	2,347	2	1.00	08	1,685	1	0.50	55
	32	2,393	2	1.02	06	3,006	2	1.08	01
Sepharose-BOT	2	1,052	1/0	0.22	70	996	1/0	0.19	84
	4	1,373	1	0.37	55	1,479	1	0.41	64
	8	1,670	1	0.50	41	1,519	1	0.43	62
	16	2,340	2	0.93	09	1,665	1	0.49	56
	32	2,446	2	1.08	03	2,583	2	1.60	18
Sepharose-HSA	2	2,007	1	0.72	25	2,316	2	0.96	29
	4	2,085	1	0.79	21	2,513	2	1.12	21
	8	2,168	1	0.84	17	2,880	2	1.46	06
	16	2,432	2	1.07	04	2,947	2	1.55	03
	32	2,508	2	1.11	01	3,005	2	1.60	01
Unabsorbed		2,517	2	1.00		3,027	2	1.62	

PRU = Phadebas RAST unit.

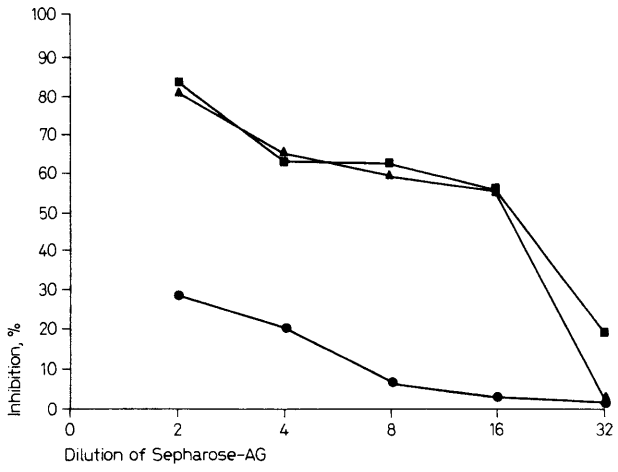


Fig. 1. Inhibition of patient IgE binding to NH_4B RAST discs by varying concentrations of Sepharose-bound antigens Sepharose-BOT (■) Sepharose-MEL (▲) and Sepharose-HSA (●).

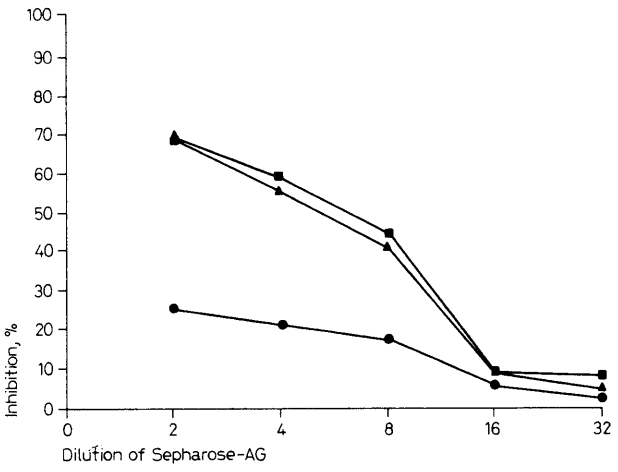


Fig. 2. Inhibition of patient IgE binding to NH_4M RAST discs by varying concentrations of Sepharose-bound antigens. Sepharose-MEL (■) Sepharose-BOT (▲) and Sepharose-HSA (●).

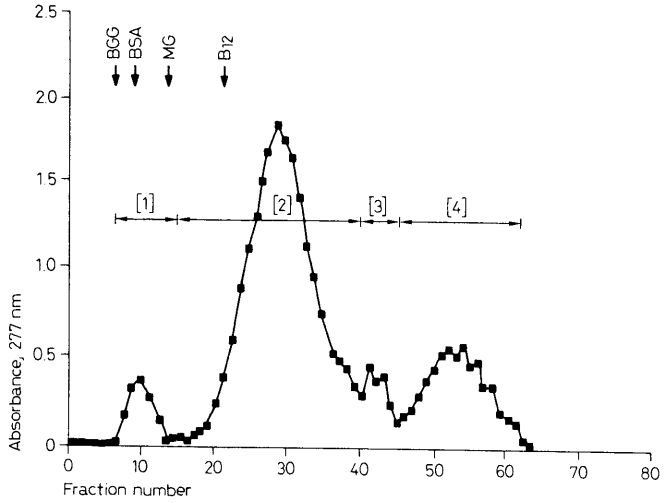


Fig. 3. Separation of proteins in the ammonium bicarbonate extract of bottlebrush (NH_4B) by Sephadex G-100 column chromatography. Arrows indicate the elution fractions of the following standards: bovine γ-globulin (BGG), bovine serum albumin (BSA), myoglobin (MG), and vitamin B_{12} (B_{12}).

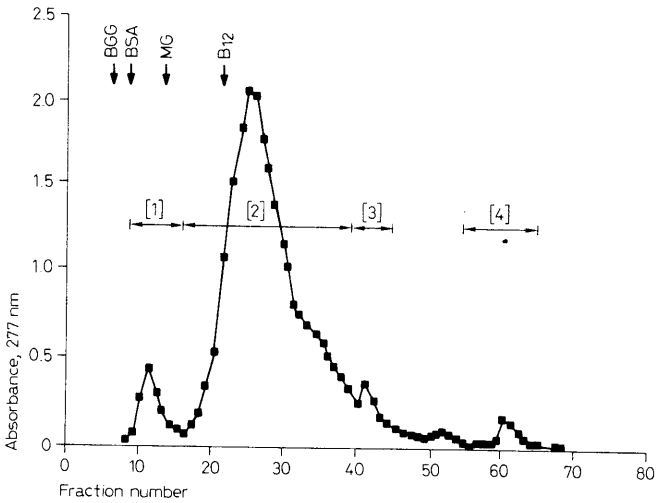
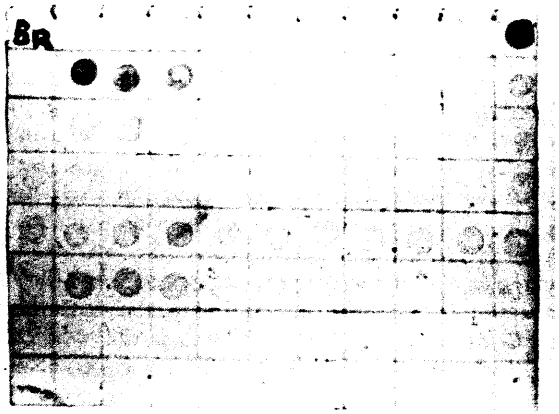


Fig. 4. Separation of proteins in the ammonium bicarbonte extract of melaleuca (NH_4M) by Sephadex G-100 column chromatography. Arrows indicate the elution fractions of the same standards as those presented in figure 3.



a

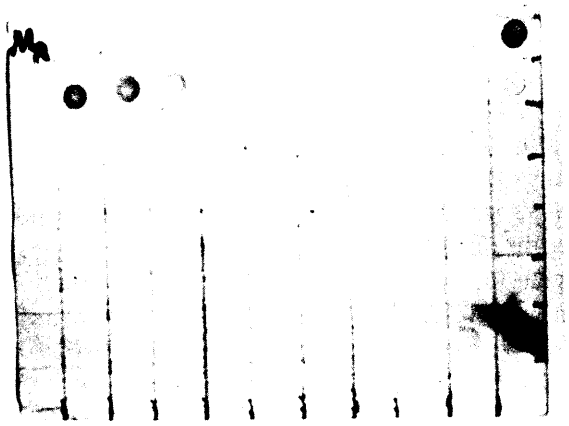
b

B_R	1	2	3	4	5	6	7			
	●	●	●	◑	◑	○	○	○	◫	◐
	◐	◑	◑	◫	○	26	27	28	29	30
	31	32	33	34	35	36	37	38	39	○
	○	◐	●	◑	◫	○	○	○	◐	◐
	◐	◑	◑	○	55	56	57	58	59	60
	61	62	63	64	65	66	67	68	69	70
	71	72	73	74	75	76	77	78	79	80

Fig. 5. Immunoblot analysis of the bottlebrush (NH_4B) fractions obtained with gel filtration. **a** Photograph. **b** Schematic representation of **a** with intensity of color reactions designated for each fraction as follows: clear circle: very light staining; circle with vertical line: light staining; circle dark on left: medium staining, left side of elution peak; circle dark on right: medium staining; right side of elution peak, dark circle: dark staining, peak fractions. Those squares designated only by a number represent fractions with no observable staining.

to 18% using NH_4B discs and from 70 to 3% using NH_4M discs (fig. 1, 2). Sepharose-BOT and Sepharose-MEL were similar in their ability to inhibit the binding of allergic patient IgE to either NH_4B or NH_4M RAST discs (fig. 1, 2). The maximum inhibitions occurred at the highest concentrations of Sepharose-AG (s) and were approximately 70% using NH_4M discs and 80% using NH_4B discs. In contrast, when Sepharose-HSA was used as a control, the RAST analysis using NH_4M and NH_4B discs showed only 25 and 29% maximal inhibition, respectively.

a

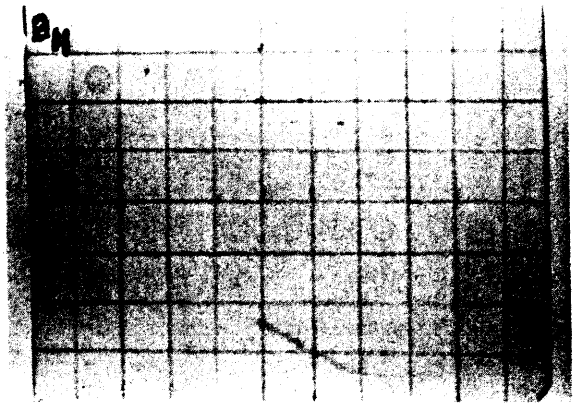


b

M_R	1	2	3	4	5	6	7	8	◐	◐
	●	●	◑	◑	◑	◐	○	○	○	◐
	○	22	23	24	25	26	27	28	29	30
	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50
	51	52	53	54	55	56	57	58	59	60
	61	62	63	64	65	66	67	68	69	70
	71	72	73	74	75	76	77	78	79	80

Fig. 6. Immunoblot analysis of the melaleuca (NH_4M) fractions obtained with gel filtration. **a** Photograph. **b** Schematic representation using symbols as in figure 5.

a



b

B_H	1	2	3	4	5	6	7	8	9	○
	◐	◐	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30
	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50
	51	52	53	54	55	56	57	58	59	60
	61	62	63	64	65	66	67	68	69	70
	71	72	73	74	75	76	77	78	79	80

Fig. 7. Allergoblot analysis of the bottlebrush (NH_4B) fractions obtained with gel filtration. **a** Photograph. **b** Schematic representation using symbols as in figure 5.

Separation of Antigenic Components by Gel Filtration

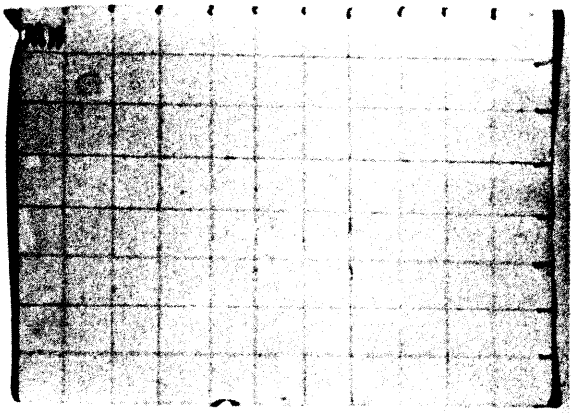
Four peaks were resolved from both antigens using gel chromatography. The fractions were designated NH_4B1–NH_4B4 and NH_4M1–NH_4M4, respectively (fig. 3, 4).

Protein Blotting of Sephadex G-100 Fractions

Peaks NH_4B1, NH_4B2, NH_4B3 and NH_4B4 were shown to have antigenic activity when incubated with rabbit antibottlebrush serum (fig. 5). Peaks NH_4M1 and NH_4M2 were also shown to contain antigenic activity when incubated with rabbit antimelaleuca serum (fig. 6). The results of the immunoblots of the G-100 fractions of NH_4B and NH_4M using rabbit antisera to the respective pollen are also represented schematically (fig. 5, 6). Each square represents a specific fraction obtained from Sephadex G-100 chromatography of the respective ammonium bicarbonate pollen extracts. Those squares designated with a circle indicate fractions containing antigenic reactivity to either rabbit antibottlebrush or antimelaleuca serum detected with peroxidase-labeled goat antirabbit IgG. Those squares designated only by number represent fractions with no observable staining.

Allergoblots of the G-100 fractions of NH_4B and NH_4M using allergic patient sera and peroxidase-labeled goat antihuman IgE demonstrated that the NH_4B1 and NH_4M1 peaks contain allergenic reactivity (fig. 7, 8).

a



b

M_H	1	2	3	4	5	6	7	8	9	○
	◐	◑	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30
	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50
	51	52	53	54	55	56	57	58	59	60
	61	62	63	64	65	66	67	68	69	70
	71	72	73	74	75	76	77	78	79	80

Fig. 8. Allergoblot analysis of the melaleuca (NH_4M) fractions obtained with gel filtration. **a** Photograph. **b** Schematic representation using symbols as in figure 5.

Molecular Weight Determinations

The molecular weights of the allergenic peaks were estimated by comparing retention times with standards run on the same column. The peaks, NH_4B1 and NH_4M1, were eluted from the column between the bovine serum albumin (67,000 daltons) and myoglobin (17,000 daltons) standards. The estimated molecular weights of NH_4B1 and NH_4M1 peaks are 50,000 and 35,000 daltons, respectively.

Discussion

Various techniques have been used to characterize bottlebrush and melaleuca pollens. TCIE analysis previously determined that the NH_4B and NH_4M extracts contain multiple antigenic components which were extensively antigenic and cross-reactive. TECIE analysis identified some of these components to be allergenic as well as antigenically cross-reactive [7]. RAST inhibition studies reported here further substantiate that NH_4B and NH_4M extracts contain cross-reactive allergenic components.

RAST inhibition analysis has greatly enhanced the detection of allergenic cross-reactivity among pollen allergens [6]. Sepharose-bound antigens, either bottlebrush or melaleuca, were able to competitively inhibit the binding of allergic patient IgE to NH_4B and NH_4M RAST discs. The variations in percent inhibition of IgE binding to NH_4B or NH_4M discs, when Sepharose-BOT or Sepharose-MEL was used as the competitor, were in the same range of variability as previously reported for duplicate samples in RAST (Ventrex Laboratories, Portland, Me). These RAST analyses, therefore, indicate that the antigenic components of the NH_4B and NH_4M extracts apparently contain some very similar, if not identical, allergenic determinants. Whether the IgE antibodies present in the RAST-positive sera that were reactive to the antigens tested were originally made in response to the cross-reactive allergenic determinants in either melaleuca or bottlebrush pollen cannot be determined from these studies. The possibility exists that the IgE antibodies in the allergic patient sera were instead made against a similar or identical allergenic determinant found in a different but allergenically related tree pollen.

Gel filtration analysis of the NH_4B and NH_4M pollen extracts identified 4 peaks. Allergoblot analysis of gel filtration fractions found that the NH_4B1 and NH_4M1 peaks contain allergenic reactivity and have estimated molecular weights of 50,000 and 35,000 daltons, respectively. These fractions of NH_4B_1 and NH_4M_1 are currently being purified electrophoretically and the individual components analyzed for allergenic reactivity. The 3 peaks not found to be allergenic would not be expected to be, because they eluted past the molecular weight range of most allergens, which is 10,000–60,000 daltons [12].

The Allergoblot technique we developed was used to detect allergen-specific IgE in patient sera. Allergoblot can detect IgE binding to allergens which are in very low concentration, such as gel filtration fractions. RAST has previously been reported to be the most convenient technique to identify these fractions [13]. Allergoblot may be performed more rapidly and less expensively than RAST techniques without loss

of sensitivity [14]. This technique has the potential for use as an initial screen for detecting possible allergic reactivity of patients to many different allergen extracts. When used prior to the administration of skin tests, this screen might avoid possible adverse reactions. Present studies are directed toward a comparison and evaluation of Allergoblot as a possible screen for allergen testing.

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References

1 Chakrabarty, S.; Lowenstein, H.; Ekramoddoullah, A.; Kisil, F.; Sehon, A.: Detection of cross-reactive allergens in Kentucky Bluegrass pollen and six other grasses by crossed radioimmunoelectrophoresis. Int. Archs Allergy appl. Immun. *66:* 142–157 (1981).

2 Halmepuro, L.; Vuontela, K.; Kalimo, K.; Bjorksten, F.: Cross-reactivity of IgE antibodies with allergens in birch pollen, fruits and vegetables. Int. Archs Allergy appl. Immun. *74:* 235–240 (1984).

3 Leiferman, K.; Gleich, G.: The cross-reactivity of IgE antibodies with pollen allergens. Part I. J. Allergy clin. Immunol. *58:* 129–138 (1976).

4 Leiferman, K.; Gleich, G.; Jones, R.: The cross-reactivity of IgE antibodies with pollen allergens. Part II. J. Allergy clin. Immunol. *58:* 140–148 (1976).

5 Viander, M.; Valovirta, E.; Vanto, T.; Koivikko, A.: Cross-reactivity of cat and dog allergen extracts. Int. Archs Allergy appl. Immun. *71:* 252–260 (1983).

6 Bernstein, I.; Perera, M.; Gallagher, J.; Michael, G.; Johansson, A.: In vitro cross-allergenicity of major aeroallergenic pollens by the radioallergosorbent technique. J. Allergy clin. Immunol. *57:* 141–152 (1976).

7 Stanaland, B.; White, R.; Gennaro, R.; Bausher, M.; Klotz, S.; Sweeney, M.: Isolation and characterization of cross-reactive allergenic components in *Callistemon citrinis* and *Melaleuca quinquenervia* pollen by trans-blot enzyme-linked crossed immunoelectrophoresis. Int. Archs Allergy appl. Immun. *80:* 278–284 (1986).

8 Rose, N.; Friedman, H.; Manual of clinical immunology; 2nd ed. (American Society for Microbiology, Washington 1980).

9 Gleich, G.; Larson, J.; Jones, R.; Baer, H.: Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent test. J. Allergy clin. Immunol. *53:* 158 (1974).

10 Ceska, M.; Eriksson, R.; Varga, J.: Radioimmunosorbent assay of allergens. J. Allergy clin. Immunol. *49:* 1–9 (1972).

11 Bio-Rad Immun-Blot Assay Kit Instruction Manual GAR-HRP (Goat Anti-Rabbit IgG Horseradish Peroxidase Conjugate) (Bio-Rad Laboratories, Richmond).

12 Chakrabarty, S.; Ekramoddoullah, A.; Kisil, F.; Sehon, A.: Allergenic relationships of two different antigens of Kentucky Bluegrass pollen. Int. Archs Allergy appl. Immun. *66:* 13–20 (1981).

13 Aukrust, L.; Grimmer, O.; Aas, K.: Demonstration of distinct allergens by means of immunological methods. Int. Archs Allergy appl. Immun. *57:* 183–192 (1978).

14 Sweeney, M.; White, R.; Stanaland, B.: Evaluation of a new in-vitro test for antigen specific IgE. Fla Scient. *50:* 30 (1987).

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