

Isolation and Characterization of Cross-Reactive Allergenic Components in *Callistemon citrinis* and *Melaleuca quinquenervia* Pollen by Trans-Blot Enzyme-Linked Crossed Immunoelectrophoresis

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Abstract. Antigenic extracts obtained from *Callistemon citrinis* (bottlebrush) and *Melaleuca quinquenervia* (melaleuca) pollen were analyzed by crossed immunoelectrophoresis (CIE), tandem-crossed immunoelectrophoresis (TCIE), and trans-blot enzyme-linked crossed immunoelectrophoresis (TECIE). CIE analysis detected 14 and 12 antigenic components in bottlebrush and melaleuca, respectively. TCIE analysis of bottlebrush pollen, employing rabbit anti-melaleuca serum in the intermediate gel, demonstrated that all of the antigenic components detected were cross-reactive. Similar analysis of melaleuca using rabbit anti-bottlebrush serum in the intermediate gel also showed that all of the antigenic components were cross-reactive. At least three bottlebrush and two melaleuca cross-reactive pollen components were demonstrated to be allergenic by TECIE analysis of CIE and TCIE gels.

Introduction

Increased interest in the study of allergen extracts and the determination of which components of these extracts are allergenic has occurred during the past 10 years [15]. This interest has been stimulated by the development of new immunochemical techniques to facilitate the separation of antigens and the determination of which antigenic components are actually allergenic [4]. Unfortunately, the majority of current research in allergen characterization has been restricted to the major allergens which are widely distributed [3, 4, 6, 11]. Allergens found only in specific areas and antigens not commonly thought to be responsible for allergic reactions have not been well studied.

Allergenic cross-reactivity among pollens of related species of trees and grasses is documented [4]. Individuals allergic to one grass or tree pollen may also suffer from allergic reactions following exposure to other grasses or trees not indigenous to the locale where the sensitizations occurred [8].

Callistemon citrinis (bottlebrush) and *Melaleuca quinquenervia* (melaleuca) are in the family Myrta-

ceae. They were originally imported from Australia and are now found throughout Florida. For many years, allergists have speculated that bottlebrush and melaleuca pollens 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 the clinical observation.

The study reported here evaluated the cross-antigenicity between bottlebrush and melaleuca pollen using crossed immunoelectrophoresis (CIE) and tandem-crossed immunoelectrophoresis (TCIE). Trans-blot enzyme-linked crossed immunoelectrophoresis (TECIE) analysis developed in our laboratory was utilized to determine which, if any, of the cross-reactive antigenic components are allergenic. TECIE unlike crossed radio immunoelectrophoresis (CRIE) does not involve the use of autoradiography which involves a lengthy development time after incubation with sera from allergic patients and ¹²⁵I labeled anti-human IgE antibodies.

Materials and Methods

Allergen Extracts

Pollen from melaleuca and bottlebrush trees was 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. Four individual extractions were performed on the defatted pollens for 48 h at 4°C (fig. 1). The first three extractions were water extractions with 200 ml distilled water at pH 7.00, 10.00 and 2.00, adjusted with 1.0 M HCl and 1.0 M NaOH. The fourth extraction was performed in 200 ml 0.005 M ammonium bicarbonate at pH 7.8. The incubated extracts were centrifuged at 40,000 g for 20 min. The resultant supernates were filtered using 0.65, 0.45, and 0.22- μ m millipore filters. Filtered material was lyophilized and stored at -20°C until use. Four different extracts of each pollen were produced (fig. 1).

Rabbit Antisera

Antisera toward bottlebrush and melaleuca pollens were prepared in rabbits. Intramuscular injections were performed in the flanks every 2 weeks for 6 weeks, then once a month thereafter. The first injection contained 20 mg of the crude defatted pollen triturated into a solution of 1 ml of phosphate-buffered saline (PBS). All subsequent injections contained 2 mg of the ammonium bicarbonate extracted material (NH_4B or NH_4M) in a volume of 1 ml containing equal parts of Freund's complete adjuvant and PBS. Subcutaneous injections, performed at various times between adjuvant injections, used 0.01 ml of a solution containing 2 mg of extracted antigen per ml PBS. Rabbits were bled 7–9 days following each adjuvant injection. Sera for each antigen were collected from at least two rabbits and pooled for immunochemical analysis.

Human Serum

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 using a grading system of Rose and Friedman [14]. The concentration of each extract used for skin testing was a 10^{-3} -fold dilution (0.1% w/v) typically recognized as a concentration which gives maximum sensitivity and least nonspecificity in intradermal skin testing. Patients were considered nonallergic if skin test results were 0 and their IgE levels were between 20 and 50 IU.

Radioallergosorbent Test (RAST) of Pollen Extracts

Allergosorbent discs (solid-phase allergen) were prepared by coupling each of the four pollen extracts of melaleuca and bottlebrush to cyanogen-bromide-activated Whatman No. 44 filter paper discs and used for RAST testing [7]. The concentration of extracts used for coupling to discs was varied to determine the maximum specific binding [7], and for each extract analyzed the concentration which produced maximum binding was approximately 5 mg/25 ml. Activated discs were incubated for 3 h with 100 μ l of either pooled allergic or nonallergic patient sera. Pooled allergic sera consisted of sera obtained from approximately 8 patients skin test positive to the allergens. The nonallergic pool consisted of sera from patients whose IgE levels were between 20 and 50 IU/ml and who

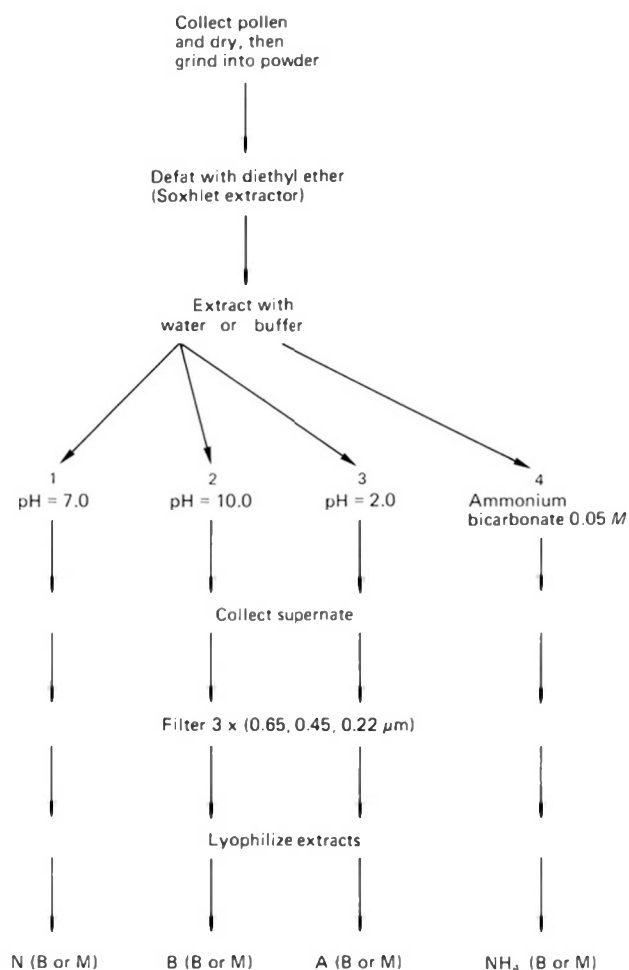


Fig. 1. Preparation of antigenic extracts from bottlebrush and melaleuca pollen. Extracts labeled as follows: N = neutral, B = basic, A = acidic, NH_4 = ammonium bicarbonate, (B or M) = bottlebrush or melaleuca extract.

demonstrated no skin test reactivity to bottlebrush and melaleuca. The discs were washed and incubated overnight in 50 μ l of ^{125}I -labeled goat anti-human IgE. Counts per minute (cpm) were then measured in a Picker (Spectroscale 4R) gamma counter. All discs were analyzed in duplicate.

Crossed Immunoelectrophoresis

CIE was performed using 7.5 $\times$ 7.5 cm glass plates covered with Gel-Bond (Bio-Rad), and 1% agarose dissolved in 0.02 M Veronal buffer, pH 8.6, containing 0.005 M calcium lactate and 0.05 M sodium azide [12, 16]. Samples of 5 μ l each of either crude bottlebrush or melaleuca pollen extract (10 mg/ml) were subjected to electrophoresis in the first dimension at room temperature for 25 min at 10 V/cm. The second dimension electrophoresis was performed at room temperature for 16–20 h at 2 V/cm with 20 μ l of rabbit anti-serum/ cm^2 gel. Gels were deproteinized for 24 h in PBS, pressed under filter paper, dried, and stained with Coomassie Brilliant Blue R-250 (Eastman) [9].

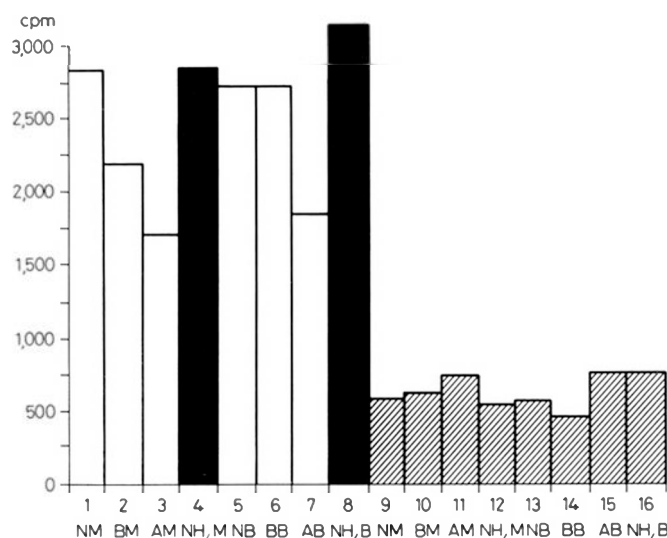


Fig. 2. RAST analysis of bottlebrush and melaleuca pollen extracts. Solid bars (black = NH_4B and HN_4M ; white = all other extracts) represent allergens analyzed with pooled allergic patient sera. Hatched bars represent allergens analyzed with non-allergic patient sera. The cpm values represent the maximum IgE binding obtained. Figures represent extract number.

Tandem-Crossed Immunoelectrophoresis

TCIE was performed with two gel sections containing different antisera [2, 10]. This technique was used to determine which antigens were cross-reactive. TCIE was performed as previously described for CIE with the addition of 20 μl of antiserum/ cm^2 in the intermediate gel.

Trans-Blot Enzyme-Linked Crossed Immunoelectrophoresis

TECIE is trans-blot electrophoresis applied to CIE and tandem-CIE gels, as previously described [13], with the following modifications: washed nitrocellulose membranes were incubated in a 1:5 dilution of allergic human serum for 15–18 h. Allergic human sera consisted of a pool obtained from at least eight patients who demonstrated skin test reactions to both allergens. These membranes were subsequently incubated for 3 h in a 1:3,000 dilution of goat anti-human IgE (ϵ -chain-specific) horseradish peroxidase labeled serum. The sheets were developed according to the Bio-Rad Immuno-Blot assay [5].

Results

RAST Analysis

RAST analysis of bottlebrush and melaleuca extracts demonstrated binding of IgE to all eight extract types using allergic patient sera (fig. 2). The acid extracts of bottlebrush and melaleuca bound a maximum of between 1,700 and 1,800 cpm using serum from allergic patients. These cpm values for the acid

extracts were 400–1,200 cpm less than the maximum cpm values obtained with any other extract type. Ammonium bicarbonate extracts of both bottlebrush and melaleuca pollen maximally bound between 2,800 and 3,200 cpm which were equal or greater cpm than any other extract type. RAST analysis of the pollen extracts using nonallergic patient sera showed very low levels of IgE binding because cpm values ranged between 500–700 for all extract types.

CIE Analysis

The patterns obtained using hyperimmune rabbit antisera to the respective pollen in CIE analysis of NH_4B and NH_4M antigenic extracts are shown in figures 3 and 4. In all cases, the anode is represented on the left side of the gel while the cathode is on the right. Many antigenic components were detected after protein staining of the CIE plates. Schematic drawings of the CIE patterns with individually numbered precipitates show the presence of at least 14 and 12 antigenic components in NH_4B and NH_4M extracts, respectively (fig. 3, 4).

TCIE Analysis

Patterns obtained with TCIE analysis of NH_4B and NH_4M are shown in figures 5 and 6. All of the visualized antigenic components of NH_4B precipitated in the intermediate gel which contained antiserum to melaleuca pollen (fig. 5). TCIE analysis of NH_4M also detected precipitin bands in the intermediate gel when analyzed with antisera to bottlebrush pollen in the tandem gel (fig. 6).

TECIE Analysis

Analysis of the CIE patterns of NH_4B and NH_4M extracts utilizing TECIE is shown in figures 7 and 8. IgE from allergic patient sera was shown to be reactive to precipitin bands 2, 3 and 4 of the CIE of NH_4B since these were the only bands that developed after incubation with horseradish peroxidase labeled goat anti-human IgE (fig. 7). Similar analysis of the CIE of NH_4M demonstrated that precipitin bands 1 and 2 bound IgE from allergic patient sera (fig. 8).

Analysis by TECIE of the TCIE pattern of NH_4B extract demonstrated that precipitin bands 2, 3 and 4, in the intermediate gel developed with rabbit anti-melaleuca serum, also bind IgE (fig. 9). Similar analy-

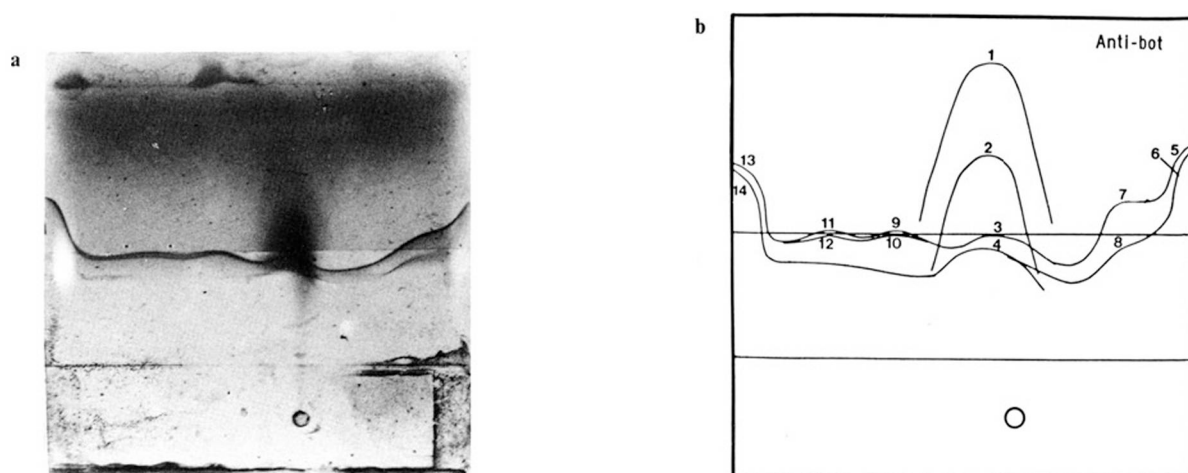


Fig. 3. CIE NH_4B against hyperimmune rabbit antisera to bottlebrush. **a** Photograph. **b** Schematic representation of NH_4B CIE.

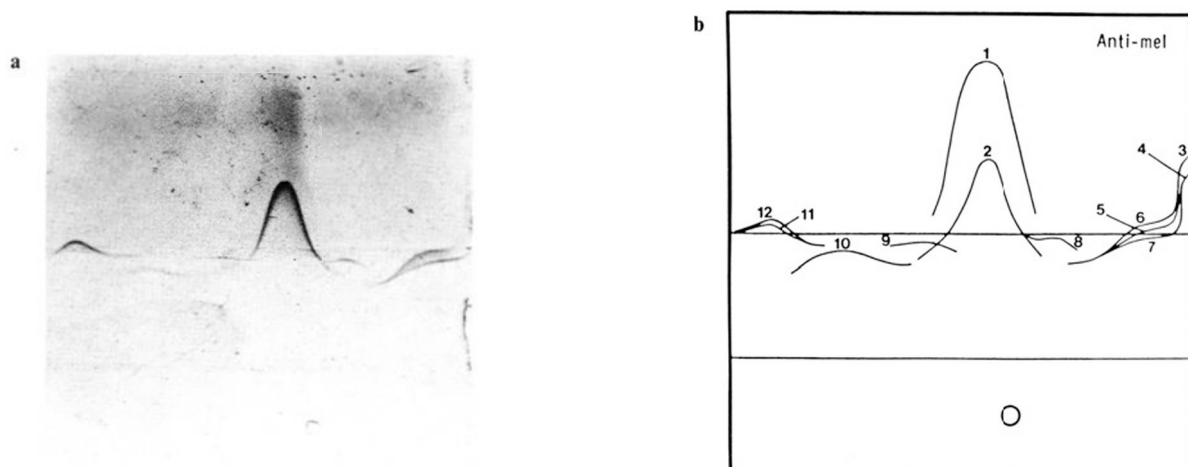


Fig. 4. CIE NH_4M against hyperimmune rabbit antisera to melaleuca. **a** Photograph. **b** Schematic representation of NH_4M CIE.

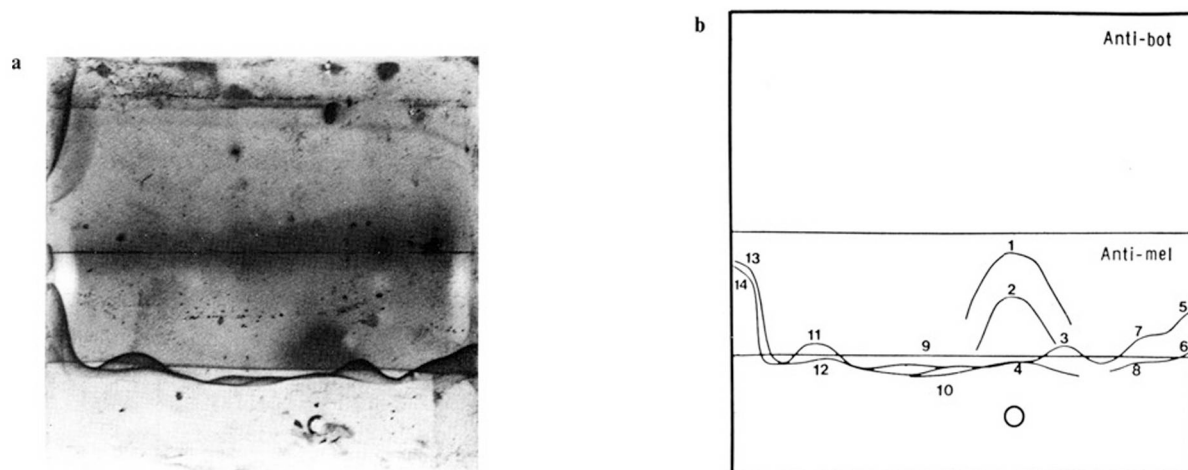


Fig. 5. TCIE NH_4B against hyperimmune rabbit antisera to melaleuca in the tandem gel and bottlebrush in the second dimension gel. **a** Photograph. **b** Schematic representation of TCIE of NH_4B with numeration as in figure 3.

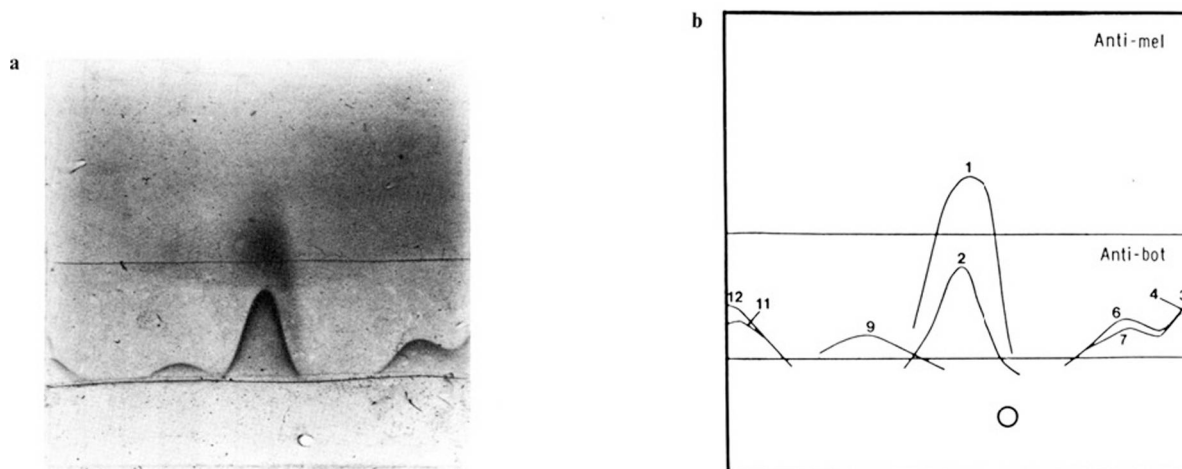


Fig. 6. TCIE NH_3M against hyperimmune rabbit antisera to bottlebrush in the tandem gel and melaleuca in the second dimension gel. **a** Photograph. **b** Schematic representation with numeration as in figure 4.

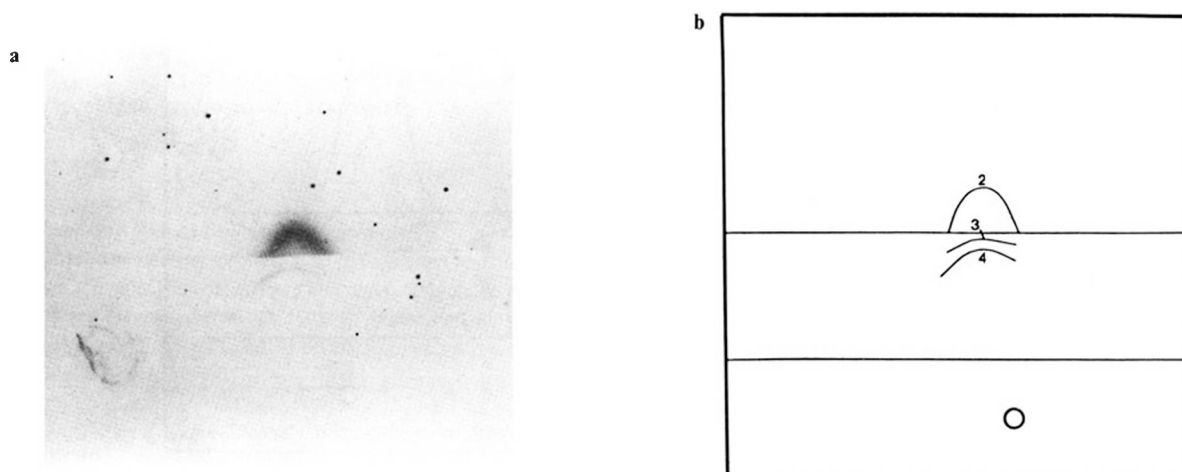


Fig. 7. TECIE of the CIE of NH_3B . **a** Photograph. **b** Schematic representation with numeration as in figures 3 and 5.

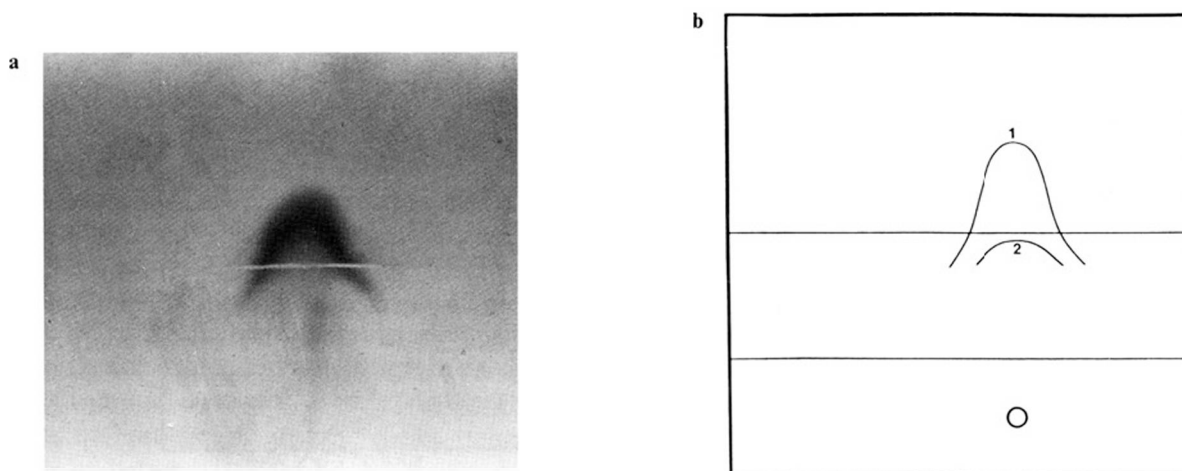


Fig. 8. TECIE of the CIE of NH_3M . **a** Photograph. **b** Schematic representation with numeration as in figures 4 and 6.

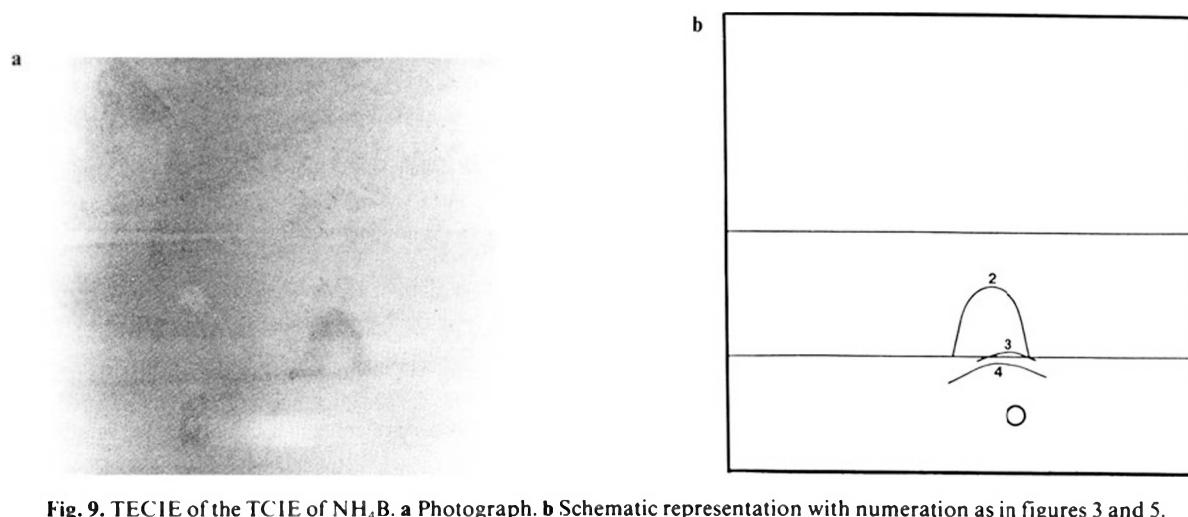


Fig. 9. TECIE of the TCIE of NH_4B . **a** Photograph. **b** Schematic representation with numeration as in figures 3 and 5.

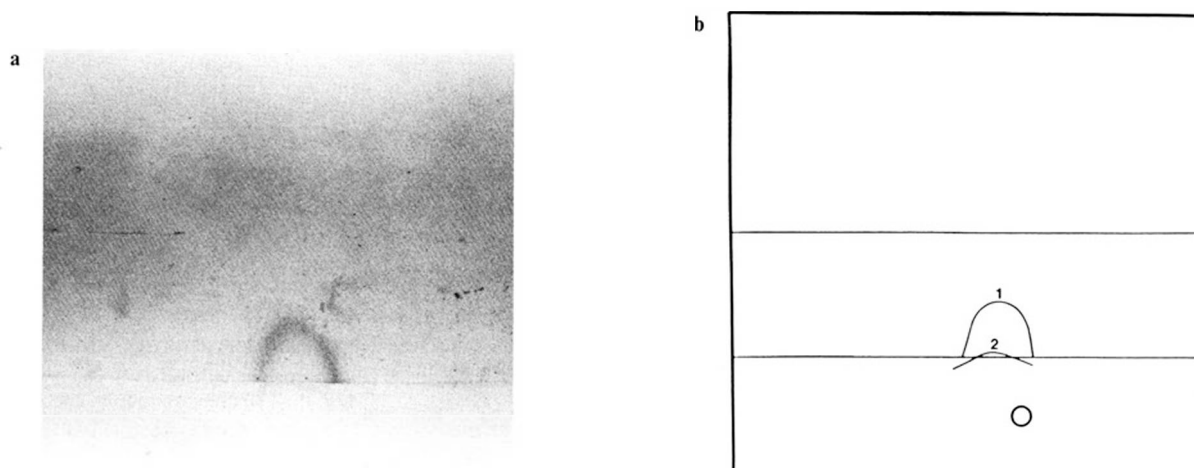


Fig. 10. TECIE of the TCIE of NH_4M . **a** Photograph. **b** Schematic representation with numeration as in figures 4 and 6.

sis of NH_4M , detected that precipitin bands 1 and 2, which occurred in the intermediate gel containing rabbit anti-bottlebrush serum, bound IgE from allergic patient sera (fig. 10).

Discussion

RAST analysis of pollen extracts demonstrated that NH_4B and NH_4M pollen extracts contained as much or greater allergenic reactivity than any of the other extract types when using specific allergic patient sera. These extracts, NH_4B and NH_4M , were therefore selected for further characterization.

The identification of antigens within a complex mixture of components, such as pollen extracts, has been greatly facilitated by CIE and variations of this technique [8]. CIE analysis of the NH_4B and NH_4M extracts utilizing antibodies raised in rabbits demonstrated that the two pollens contain multiple antigenic components. TCIE analysis indicated that these pollen antigens are cross-reactive because precipitin bands were detected in the tandem gel containing sera to the opposite pollen, therefore demonstrating a strong reaction with antisera raised to the other antigen.

Trans-blot enzyme linked analysis of CIE and TCIE gels demonstrated that at least three bottlebrush

and two melaleuca cross reactive pollen components were allergenic. The use of an enzyme-linked anti-human IgE in detection of allergic components in CIE and TCIE patterns trans-blotted to nitrocellulose membranes has not been previously reported. This technique is very sensitive and has a 50-pg detection limit [5]. TECIE detects allergenic bands in immunoprecipitates faster than CRIE. Development time after incubation with anti-human IgE horseradish peroxidase labeled antibodies is less than 1 h. The potential of this technique for aid in rapid detection of allergenic components in CIE analysis is obvious.

Problems have been reported to occur with the detection of allergenic components in precipitin bands. It has been previously demonstrated that precipitates may contain trace amounts of allergens due to coprecipitation if they are positioned in areas enclosed by precipitates of these allergens [1]. Therefore, precipitin bands 3 and 4 using TECIE of the NH₄B extract might contain enough trace amounts of precipitin band 2 to bind IgE and, therefore, appear allergenic. Similar examination of NH₄M trans-blot patterns indicated that band 2 might contain trace amounts of band 1 and, therefore, bind IgE and appear allergenic.

Distinct antigenic components of two different pollen antigenic extracts may carry identical allergenic determinants which causes IgE cross-reactivity [1]. These antigenic components of bottlebrush and melaleuca appeared to have a different overall structure when analyzed by CIE; but, due to the extensive cross-reactivity observed by TCIE, the actual antigenic determinants which make up the antigenic components of these two pollen extracts appear to be very similar, if not identical. The question still remains whether or not the allergenic components identified by TECIE are actually cross-reactive. RAST inhibition analysis, which is presently under study, could possibly provide further substantiation of allergenic cross-reactivity.

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