

Brief communication

Polymerized tree pollen antigens

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Aqueous extracts of tree pollen were partially purified and polymerized with methods previously established for preparation of ragweed and grass polymers. The polymerized tree preparations were antigenic as demonstrated by ability to elicit immediate-type skin reactivity in humans and to induce an immune response in rabbits. The polymerized tree antigen was 100- to 10,000-fold less skin reactive than monomer tree antigen in tree pollen-sensitive patients but both preparations had similar antigenicity in rabbits. These results demonstrate that polymerized tree antigens can be prepared and should have the therapeutic potential already demonstrated for polymerized ragweed preparations.

A series of studies have described the polymerization of ragweed antigen E,¹ the physical and immunologic characteristics of polymerized antigen E, which include reduced allergenicity and retained antigenicity,² and the proposed explanation for such important features of this modified allergen.³ Subsequent clinical studies demonstrated safety and efficacy of polymerized antigen E.⁴ Later studies utilized a partially purified ragweed preparation containing a more complete spectrum of ragweed antigens. This was demonstrated by an IgG immune response of humans to ragweed antigens K and Ra3 in addition to antigen E after therapy with polymerized ragweed.⁵ The clinical efficacy of such a preparation after 1 yr of therapy was demonstrated⁶ and then extended to 2 yr of therapy.⁷

The ability to give significantly larger doses of polymerized ragweed (as compared with monomeric ragweed) in a shorter course of therapy has been demonstrated.⁸ Recently, a multi-institutional study of polymerized ragweed therapy in varying geographic locations has been completed.⁹ This study showed

clinical efficacy, IgG antibody responses, and safety in populations in four cities.

The preparation of polymerized grass pollen has been reported¹⁰ and clinical trials are in progress.

Allergen immunotherapy for seasonal pollinosis requires primarily ragweed, grass, and tree allergens. This report describes the preparation of polymerized tree allergens, their similarity to polymerized grass and ragweed allergens in chromatographic analyses, and reduced allergenicity.

METHODS AND MATERIALS

Preparation of polymer

Ether extraction. Pollens were obtained from Hollister-Stier Laboratories (Spokane, Wash.). Two grams of each of the following tree pollens were mixed: white hickory, white ash, hard maple, American elm, cottonwood, and red oak. These pollens were defatted five times by 250 ml ether at room temperature with the use of a magnetic stirrer. The pollen grains were extracted several times with ether and dried overnight.

Aqueous extraction. The defatted pollen grains were extracted with 120 ml isotonic phosphate-buffered saline (PBS), pH 7.2, at 4° C for 24 hr with the use of a magnetic stirrer. The preparation was centrifuged at $17,000 \times g$ for 1 hr and the precipitate was discarded.

Ammonium sulfate precipitation. To 95 ml crude extract at 4° C, 58 gm ammonium sulfate were added, stirring constantly for 3 hr. The resulting precipitate was sedimented at 4° C and the supernate discarded. The precipitate was dissolved in 30 ml PBS. The solution was dialyzed at 4° C against five changes of 5,000 ml PBS, at which time the

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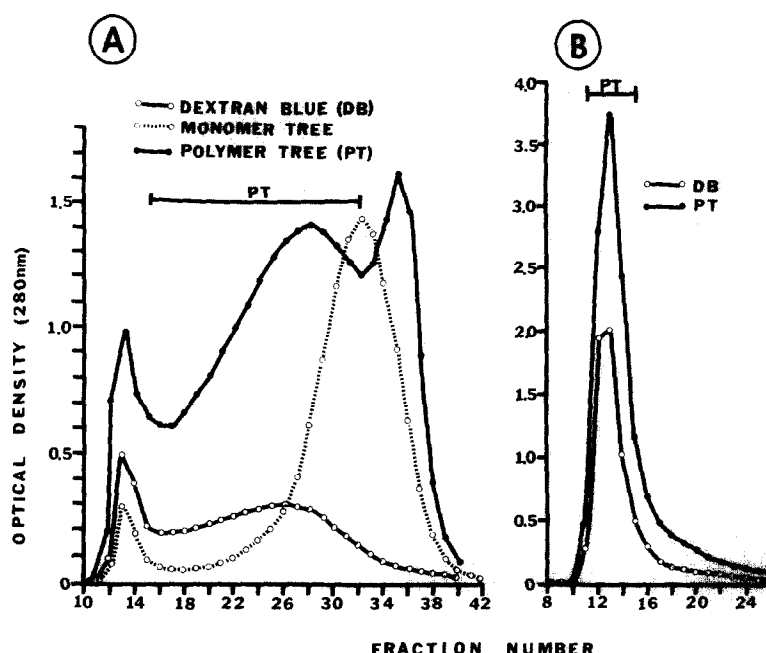


FIG. 1. A, Sephadex 4B column fractionation of dextran blue (DB), monomer tree, and monomer tree treated with glutaraldehyde to form polymer tree (PT). The line (—) indicates the fractions of PT combined for further studies of polymer. B, Sephadex G200 fractionation of dextran blue (DB) and polymerized tree (PT). The line (—) indicates the proteins in excluded fractions combined to form the final PT product.

dialysate was free of ammonium sulfate. The contents of the dialysis bag were further purified by chromatography on Sephadex G-15 column (Pharmacia) and the excluded portion by Sephadex G-15 was dialyzed against PBS and was termed "monomer tree" (MT).

Polymerization procedure

For each 10 mg MT, 0.625 ml 1:50 glutaraldehyde (25% grade II; Sigma Chemical Co., St. Louis, Mo.) was added. The polymerization of 20 to 30 mg MT was carried out at room temperature for 4 hr with the use of a magnetic stirrer. The preparation was treated with glycine to stop further polymerization with the use of a ratio of 50 M of glycine to 1 M of glutaraldehyde. The glutaraldehyde-treated mixture was applied to a Sephadex 4B (Pharmacia) column and fractionated by use of a 0.1 M tris and 0.15 M NaCl buffer, pH 7.9. The column had been calibrated previously with dextran blue to obtain exclusion and inclusion profiles. Fractions obtained were evaluated for protein content with the use of a Beckman DU spectrophotometer at 280 nm. The fractions included by the Sephadex 4B column were combined. The polymerized tree antigen (PT) was placed in a dialysis bag and concentrated by evaporation to 3 ml and dialyzed against tris-NaCl buffer, pH 7.9, overnight at 4° C. The PT was then applied to a Sephadex G-200 (Pharmacia) column. This column had been previously calibrated with dextran blue to determine the exclusion profile. The PT excluded by the Sephadex G-200 was the final preparation of PT. The PT was dialyzed for 48 hr against phosphate-buffered 0.15 M NaCl, pH 7.35, containing 0.5% phenol.

The PT was sterilized by filtration with the use of filter unit (0.20 U plain membrane, Nalge; Sybron Corp., Rochester, N. Y.). The final PT preparation was cultured for sterility by the microbiology laboratories of Northwestern Memorial Hospital at 48 hr and after 2 wk of culture.

Tree antigens were standardized at 280 nm. A solution of 1 O.D. unit/ml was determined to contain 0.5 mg protein/ml (done by micro-Kjeldahl, Allergy Laboratory of Ohio, Columbus, Ohio).

Cutaneous reactivity tests

Serial tenfold dilutions of allergens were prepared in PBS. These were tested intracutaneously in 10 subjects with a prior diagnosis of tree pollinosis. Skin tests were evaluated by standard criteria¹¹ and the highest dilution with a negative reaction was read as the end-point skin titration.

Tanned red blood cell passive hemagglutination (TRBC) assay

A standardized technique¹² was used for this assay. A suspension of TRBC was exposed to a MT solution containing 18 mg protein/ml. One percent of the antigen-coated TRBC was added to serial twofold dilutions of rabbit antisera. The highest dilution giving definite hemagglutination was read as the end-point titer.

Immunization of rabbits

Two rabbits received MT and two, PT. Immunization schedule was the same for MT and PT with each animal receiving 0.5 mg intravenously the first week and 0.5 mg

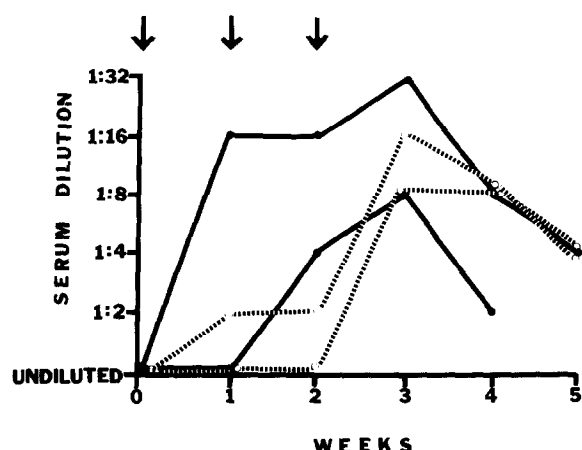


FIG. 2. Hemagglutination of tanned red blood cells coated with monomer tree antigen by sera of rabbits immunized with either monomer tree (MT) (○---○) or polymer tree (PT) (●—●) antigen. Immunizing doses of 0.5 mg are shown by arrows.

TABLE I. End-point intradermal titrations with polymerized tree and monomer tree preparations

Patient No.	Polymerized tree	Monomer tree	Difference
1	Undiluted*	1:10,000	10 ⁴
2	1:1,000	1:100,000	10 ²
3	1:10	1:100,000	10 ⁴
4	1:10	1:10,000	10 ³
5	1:10,000	1:1,000,000	10 ²
6	1:100	1:10,000	10 ²
7	1:1,000	1:100,000	10 ²
8	1:100	1:100,000	10 ³
9	1:100	1:100,000	10 ³
10	1:1,000	1:1,000,000	10 ³

*Undiluted preparations of polymer and monomer contain 1.25 mg protein/ml.

subcutaneously the second and third weeks. Animals were bled before each immunization and weekly for 3 wk after the last immunization. Sera was separated and stored at -20°C until used.

RESULTS

Polymerization of MT

The Sepharose 4B elution patterns of MT and PT are shown in Fig. 1, A. The shift in molecular size following polymerization of monomer to polymer is apparent. The glutaraldehyde-glycine peak observed in preparations of other polymerized allergens appears in the low-molecular weight region of the polymer preparation. Proteins with a molecular weight in excess of 20,000,000 are excluded by Sepharose 4B and the range of polymer molecules used for further fractionation are shown in Fig. 1, A. The PT molecules in

the Sepharose 4B fractions were combined and further fractionated through Sephadex G-200. The elution profile of PT compared with dextran blue using Sephadex G-200 is shown in Fig. 1, B. Based on these chromatographic fractionations, the final preparation of PT shown in Fig. 1, B, has a molecular weight range of between 200,000 and 20,000,000. These elution patterns were repeated in four separate polymerization experiments.

Skin-test reactivity of MT and PT

Comparisons of skin-test titrations of PT and MT are shown in Table I. The MT preparations were still reactive in dilutions 100 to 10,000 times higher than the PT preparation.

Immune response of rabbits to MT and PT

Rabbits injected with 1.5 mg of either MT or PT showed immune responses using the TRBC hemagglutination assay as shown in Fig. 2. The magnitude of the antibody responses was similar with MT or PT considering the individual variability of animals.

DISCUSSION

The results of these studies of a partially purified preparation of tree allergen (MT) demonstrate that such a preparation can be polymerized by previously described methodology to a polymerized preparation that has elution patterns similar to polymerized ragweed and grass antigens. Although the allergenicity of such a PT preparation can be demonstrated by ability to elicit an immediate skin reaction (Table I), the allergenicity of the PT preparation is sharply reduced to a degree that is equal to or greater than that for polymerized ragweed² and polymerized grass allergens. To demonstrate retained immunogenicity, the PT was compared with MT in immunized rabbits. Although aqueous pollen allergens are poorly immunogenic unless combined with complete Freund's adjuvant, our current studies in rabbits suggest that the MT and PT have approximately equal immunogenicity.

Preparations of polymerized tree allergen with sharply reduced allergenicity but retained immunogenicity should have the same potential for improved allergen immunotherapy already demonstrated for polymerized ragweed immunotherapy in a multicenter study of polymerized ragweed therapy.⁹

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