

Comparison of immune reactivity to polyvalent monomeric and polymeric ragweed antigens

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The proteins of an aqueous extract of ragweed pollen (RW) were precipitated at 90% saturation with $(\text{NH}_4)_2\text{SO}_4$, solubilized, and sieved through Sephadex G-15. The excluded fraction, termed the monomeric form (MRW), was cross-linked with glutaraldehyde to form polymerized ragweed (PRW). As compared with MRW, PRW is more immunogenic in rabbits in the production of antibody against RW antigen E (AgE). MRW and PRW resulted in equal peak reaginic responses in rabbits but the duration of reagin production was longer after immunization with MRW than with PRW. Both MRW and PRW have exposed antigenic determinants of AgE but PRW had approximately $\frac{1}{3}$ the number as shown by neutralization of human antibody against RW AgE. PRW had 100-to 1,000-fold less human cutaneous reactivity than MRW. PRW may be a more easily effective therapeutic agent for human RW immunotherapy than currently used aqueous extracts and may be more readily obtainable than polymerized AgE.

Previous studies were initiated to determine if a polymerized form of ragweed antigen E (AgE) would have reduced allergenicity but retained immunogenicity.^{1, 2} The results showed that the major allergen of ragweed, AgE,³ could be polymerized with glutaraldehyde and polymers of known molecular weight ranges obtained by column fractionation.¹ When compared with monomeric AgE, the polymeric AgE was at least as immunogenic as assessed by IgG antibody responses in rabbits.¹ The polymeric AgE was less allergenic in man, however, as demonstrated by decreased ability to elicit immediate-type skin reactivity or leukocyte histamine release.² These laboratory studies were followed by a clinical trial of polymerized AgE for therapy of ragweed pollinosis. The results showed that the polymer induced as great an IgG response, fewer immediate-type reactions, and equal clinical efficacy as compared with monomer.⁴ It was also demonstrated that the same therapeutic response could be obtained with significantly fewer injections.⁴

The limitations of polymerized AgE as a therapeutic agent include the technical difficulty of obtaining large amounts of AgE for general clinical use because of the extensive fractionation procedures required.³ Further, although AgE is a major allergen, it is not the only allergen in ragweed extract. Other allergens may be of great clinical significance because of their ability to diffuse more rapidly from pollens.⁵

The objectives of this study were as follows: first, to determine if a more complete fraction of ragweed extract containing an array of antigens could be polymerized and the higher molecular weight polymers fractionated; second, to discover if such a preparation would result in an equal immune response directed against an important known allergen (AgE) of the polymer in comparison with the unpolymerized (monomer) preparation; third, to determine if such a polymer would have reduced allergenicity when compared with the monomeric form; finally, to determine if an enhanced reaginic antibody response occurs with the polymer. All of these questions were considered relevant to the potential of the polymerized polymeric ragweed for application to human immunotherapy.

MATERIALS AND METHODS

Preparation of polymerized ragweed (PRW) from monomeric ragweed antigen (MRW)

Pollens were obtained from Hollister-Stier Laboratories (Spokane, Wash.). Giant and short ragweed pollens, 25 gm of each, were used. These pollens were defatted by extrac-

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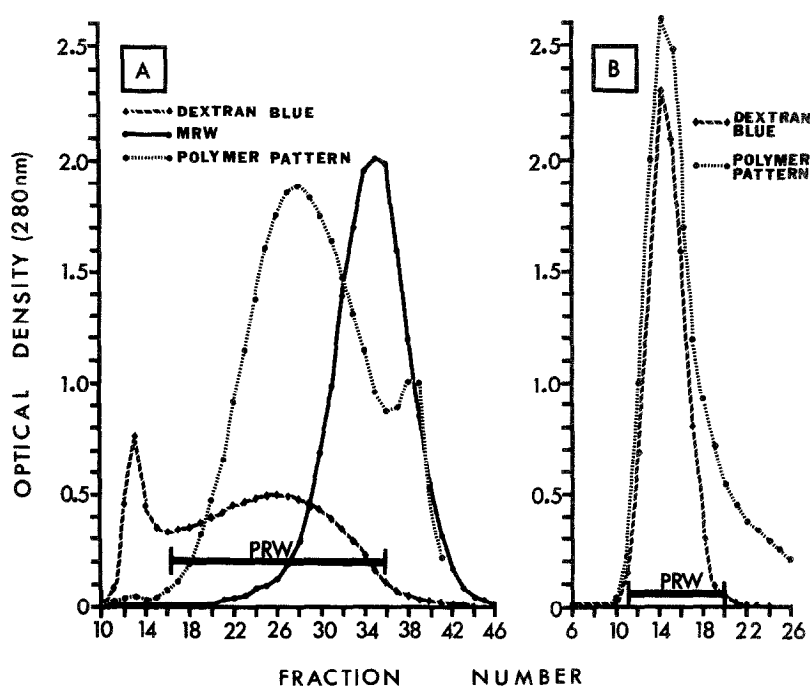


FIG. 1. A, Sepharose 4B column fractionations of preparations of ragweed (RW) prepared by precipitation with ammonium sulfate for the monomeric (MRW) and the polymeric (PRW) forms produced by treatment of MRW with glutaraldehyde. B, Column fractionation of ragweed polymers through Sephadex G-200 compared with dextran blue. The excluded fractions were pooled to form the final preparation of PRW.

tion with 1,000 ml/ethyl ether at room temperature with the use of a magnetic stirrer. The pollen grains were extracted several times with ethyl ether anhydrous and dried overnight. The defatted pollen grains were extracted with 500 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.

To 440 ml crude extract at 4° C, 268 gm ammonium sulfate was added with constant stirring which was then continued for 4 hr. The resulting precipitate was sedimented at 4° C and the supernate discarded. The precipitate was dissolved in 100 ml PBS. The solution was dialyzed with the use of dialysis membrane (Union Carbide) at 4° C against 5 changes of 5,000 ml PBS at which time the dialysate was free from ammonium sulfate. The contents of the dialysis bag were centrifuged to remove a small amount of precipitate and the supernatant solution was further purified by chromatography on a Sephadex G-15 column. Sephadex G-15 columns measured 5×60 cm, and isotonic PBS was the eluting fluid. The excluded portion of Sephadex G-15 was the monomer ragweed (MRW). Protein concentration of MRW was determined by both the biuret technique and spectrophotometrically at 280 nm with the use of a Gilford 250 spectrophotometer.

For each 10 mg MRW, 0.625 ml 1:50 glutaraldehyde (25% grade II, Sigma Chemical Company) was added. The polymerization of 20 to 30 mg MRW 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 moles of glycine to one mole of glutaraldehyde. The glutaraldehyde-treated mixture was applied to a Sepharose 4B (Pharmacia) column and fractionated with 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 Gilford 250 spectrophotometer at 280 nm. The fractions included by the Sepharose 4B column were combined. The polymerized ragweed (PRW) was placed in a dialysis bag, concentrated by evaporation to 4 ml, and dialyzed against tris-NaCl buffer, pH 7.9, overnight at 4° C. The PRW 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 fraction excluded by the Sephadex G-200 was designated PRW. Preparations of MRW and PRW were stored at -20° C until use in immunization procedures. For human use, both MRW and PRW were dialyzed for 48 hr against phosphate-buffered 0.15 M NaCl, pH 7.3, containing 0.5% phenol.

Both MRW and PRW were sterilized by filtration with the use of a filter unit (0.20μ plain membrane, Nalge, Sybron Corp., N. Y.). They were cultured for sterility by the microbiology laboratories of Northwestern Memorial Hospital at 48 hr and following 2 wk of culture. Preparations were proved to be sterile before use in the human studies reported below.

TABLE I. Endpoint cutaneous titers with the use of serial 10-fold dilutions of monomer or polymerized ragweed in 3 subjects allergic to ragweed

Concentration of RW tested ($\mu\text{g/ml}$)*	Subject 1		Subject 2		Subject 3	
	MRW	PRW	MRW	PRW	MRW	PRW
50	ND	ND	ND	ND	ND	+++
5	ND	+++	ND	ND	ND	—
0.5	ND	—	ND	+++	+++	—
0.05	+++	—	++++	—	++	—
0.005	++	—	++	—	—	—
0.0005	—	—	—	—	—	—

RW, ragweed antigen; MRW, monomer ragweed; PRW, polymerized ragweed; ND, not done.

*Based on optical density at 280 nm with the use of a Gilford 250 spectrophotometer.

Immunization procedure

Adult albino rabbits received an initial intravenous dose of 0.8 mg of PRW or MRW. One week later and every 2 wk thereafter each rabbit received 0.8 mg of PRW or MRW delivered subcutaneously divided among 4 different sites for a total of 6 doses. Blood samples were collected prior to immunization and subsequently just prior to each booster immunization. Serum was separated and stored at -20°C until used.

Determination of rabbit antibody response against AgE following immunization with MRW or PRW

AgE (supplied by Dr. Floyd McIntyre) was labeled with ^{125}I as previously described.⁶ ^{125}I -AgE is not precipitated by 40% saturation with ammonium sulfate and a modification of the Farr⁷ technique was used to determine binding of ^{125}I -AgE by antisera. Increasing amounts of ^{125}I -AgE were added to serum samples and incubated at 37°C for 2 hr and then overnight at 4°C . Sufficient saturated ammonium sulfate was added at 4°C to produce 40% saturation. The samples were thoroughly mixed, incubated at 4°C for 2 hr, and centrifuged at $1,500 \times \text{G}$. The supernatant solution was separated from the precipitate and the percentage of added ^{125}I -AgE in the precipitate was determined and expressed as micrograms of AgE bound per milliliter at 70% antigen precipitation.

Cutaneous and passive cutaneous testing procedures in rabbits

Immediate-type, active cutaneous reactivity was evaluated by testing of animals at weekly intervals with 0.1 ml of RW AgE containing antigen in concentrations beginning at $1.0\ \mu\text{g/ml}$ and including serial 10-fold dilutions to $0.001\ \mu\text{g/ml}$. Reading of skin test titers was aided by the intravenous injection of 1.0 ml of 0.5% Evans blue dye before testing with antigen. A positive reaction was read as one giving a bluing reaction 1 cm or more in diameter. The passive cutaneous reactions were done by sensitizing normal rabbits with intracutaneous injections of serial 2-fold dilutions of rabbit sera. Seventy-two hours later, the rabbit was injected intravenously with 1 ml of 0.5% Evan's blue dye and 5 ml of 1:10 crude ragweed antigen (Hollister-

Stier). Positive reactions were determined by the same criterion used for active cutaneous titration.

Cutaneous titration (human)

Three ragweed-sensitive subjects were tested by intracutaneous injection of 0.02 ml of serial 10-fold dilutions of both PRW and MRW. Reactions were graded 1+ to 4+ positive and the endpoint titration was the highest dilution giving a wheal and erythema reaction.

Polystyrene tube radioimmunoassay

This technique has been described for use in detection of IgE antibodies against *Aspergillus fumigatus*.⁸ In the current experiments the assay was used to demonstrate that MRW and PRW had similar antigenic determinants. For these experiments the sequence of addition of reagents to polystyrene tubes included MRW or PRW, posttreatment serum from a subject previously treated with standard RW immunotherapy and ^{125}I -MRW. Because posttreatment serum was used, almost all antibody detected would be IgG. A second type of experiment was designed to demonstrate that MRW and PRW had exposed antigenic determinants of RW AgE. For these experiments, the sequence of addition of reagents to the tubes included MRW or PRW, human anti-RW, and ^{125}I -AgE. MRW and AgE were radiolabeled by the method previously described.⁹

Comparison of AgE determinants on MRW and PRW by inhibition analysis

This experiment was designed to estimate the weight ratios of MRW and PRW required to neutralize human IgG antibody against RW AgE. This technique uses 5 ng ^{125}I -AgE plus just sufficient human anti-AgE to result in maximum precipitation of AgE after bringing the mixture to 40% saturation with ammonium sulfate. Aliquots of this predetermined quantity of human anti-AgE were incubated with increasing concentrations of MRW or PRW prior to the addition of 5 ng ^{125}I -AgE and the degree of binding of the ^{125}I -AgE determined at 40% ammonium sulfate saturation. The decrease in precipitation of ^{125}I -AgE after preincubation of the antiserum with varying amounts of MRW or PRW demonstrates the degree of neutralization of antibody directed against determinants of AgE.

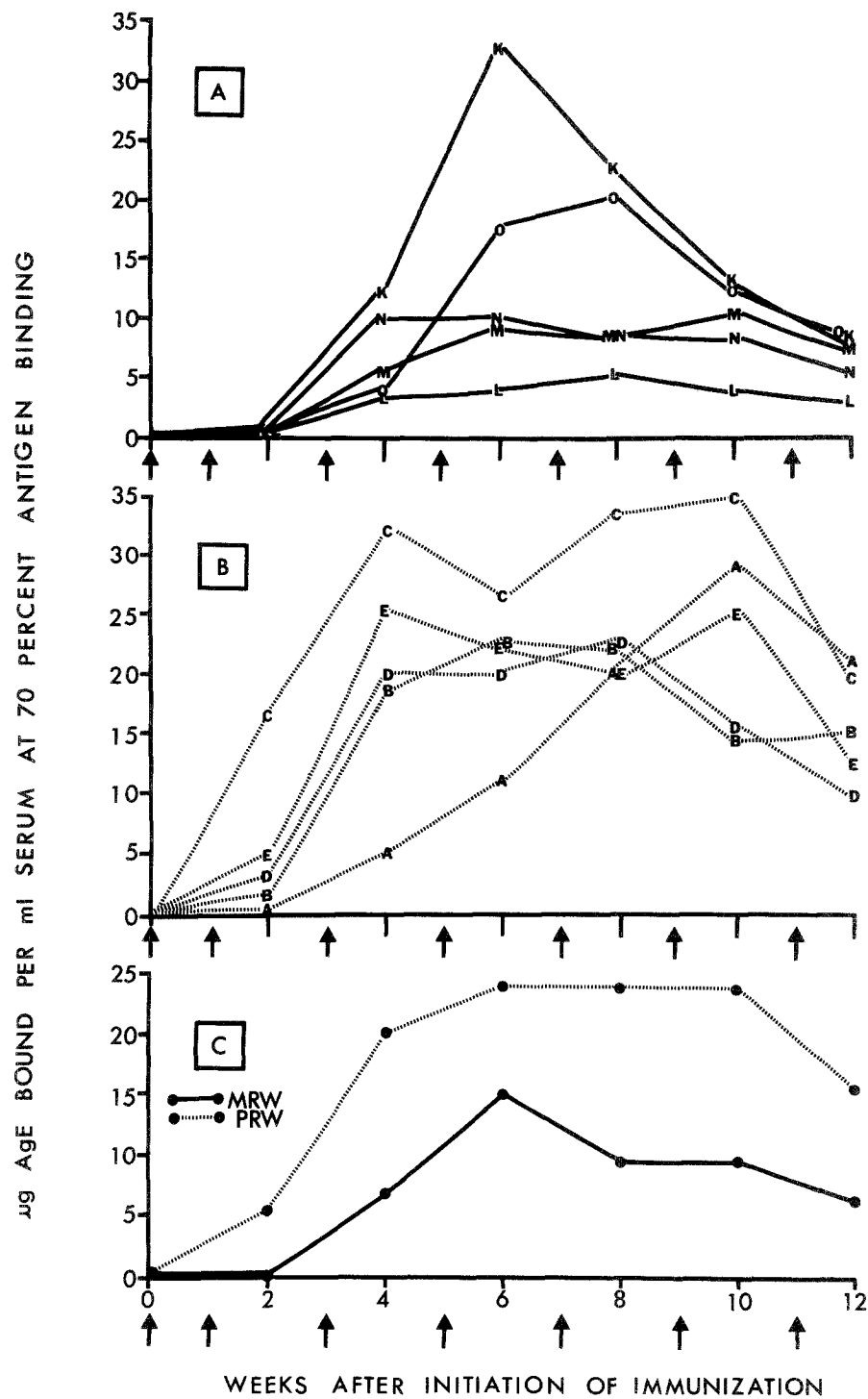


FIG. 2. A, Immune response against ragweed antigen E (RW AgE) of rabbits immunized with monomer ragweed (MRW). B, Response of rabbits immunized with PRW. C, Mean of responses of MRW and PRW groups. Arrows indicate immunizations; letters denote individual rabbits.

RESULTS

Polymerization of ragweed antigens

MRW and PRW were prepared as described in the section on materials and methods. The elution profiles of MRW and PRW with the use of Sepharose 4B are

shown in Fig. 1, A. The shift in the pattern following the treatment of MRW with glutaraldehyde demonstrates the polymerization of the MRW to provide PRW. The elution pattern of the polymerized RW from a column of Sephadex G-200 is compared with

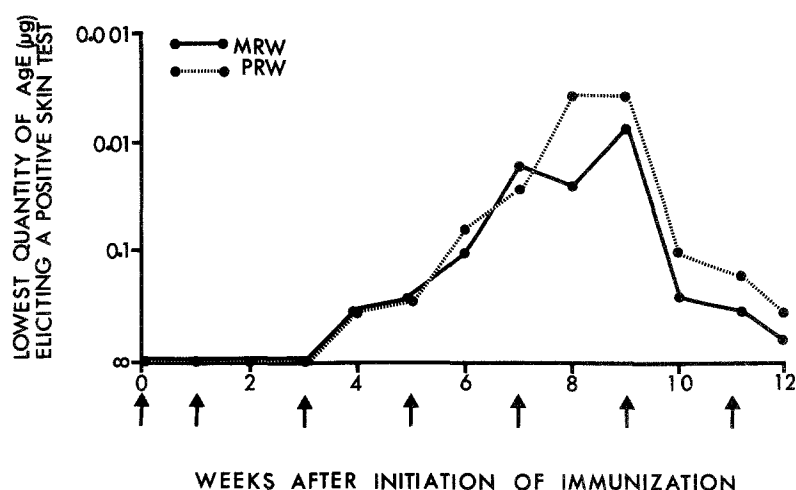


FIG. 3. Means of cutaneous endpoint titers against RW AgE of 5 rabbits immunized with either MRW or PRW. Arrows indicate immunizations.

that of dextran blue in Fig. 1, *B*. The combined fractions of the polymerized RW excluded by Sephadex G-200 shown in Fig. 1, *B* constitute the final preparation of PRW.

Immune response in rabbits

The binding capacity for ragweed AgE of rabbit sera following immunization with either MRW or PRW is shown in Fig. 2. There was an overlap of the immune response of the 2 groups of rabbits through wk 8 after onset of immunization. At wk 10 and 12, there was no overlap as the rabbits immunized with PRW all had higher serum-binding capacity for ^{125}I -AgE than did the rabbits immunized with MRW. The means of the serum-binding capacities of the rabbits in each group are shown in Fig. 2, *C*. This overall comparison shows an earlier and higher immune response against AgE in the PRW group than in the MRW group.

Reagin response in rabbits: Cutaneous titrations

The immediate-type cutaneous reactivity of rabbits to skin testing with serial 10-fold dilutions of AgE during the course of immunization with PRW and MRW is shown in Fig. 3. A slightly higher peak titer and late titer is seen in the PRW rabbits but the rate of rise and fall of the cutaneous titers in both groups is similar.

Long-latency passive cutaneous anaphylaxis

Passive cutaneous skin titers with the use of serial 2-fold dilutions of serum obtained during the immunization of rabbits with MRW or PRW are shown in Fig. 4, *A* and *B*. The skin sites were tested at 72 hr after sensitization. The means of these cutaneous

transfer titers are shown in Fig. 4, *C*. Both MRW and PRW induce reaginic antibody responses of similar magnitude which declined in both groups with continued immunization. In the limited group of rabbits immunized with MRW, there was a higher persistent long-latency reaginic antibody response (Fig. 4).

Cutaneous reactivity of MRW and PRW in ragweed-sensitive humans

Intracutaneous endpoint titrations of 10-fold dilutions of MRW and PRW standardized by optical density at 280 nm in 3 ragweed-allergic subjects are shown in Table I. In 2 subjects, the lowest concentration of PRW resulting in a positive skin test was 1,000-fold more and in one subject 100-fold more than the lowest concentration of MRW resulting in a positive skin test reaction.

Binding of human antibody to MRW and PRW

Posttreatment serum from a patient treated with standard ragweed immunotherapy was used to evaluate whether binding activity against MRW, PRW, and ragweed AgE could be demonstrated. The human serum used was known, from prior studies,⁹ to contain IgG antibody against ragweed antigens and AgE. The polystyrene tube solid-phase immunoadsorbent technique was used. Human antiragweed or control sera and finally ^{125}I -MRW or ^{125}I -AgE were added in sequence to tubes coated with either MRW or PRW. The results in Table II demonstrate that the tubes coated with MRW or PRW will bind bivalent antibody which can react with either ^{125}I -MRW or ^{125}I -AgE. The latter result demonstrates the presence of exposed AgE determinants on PRW.

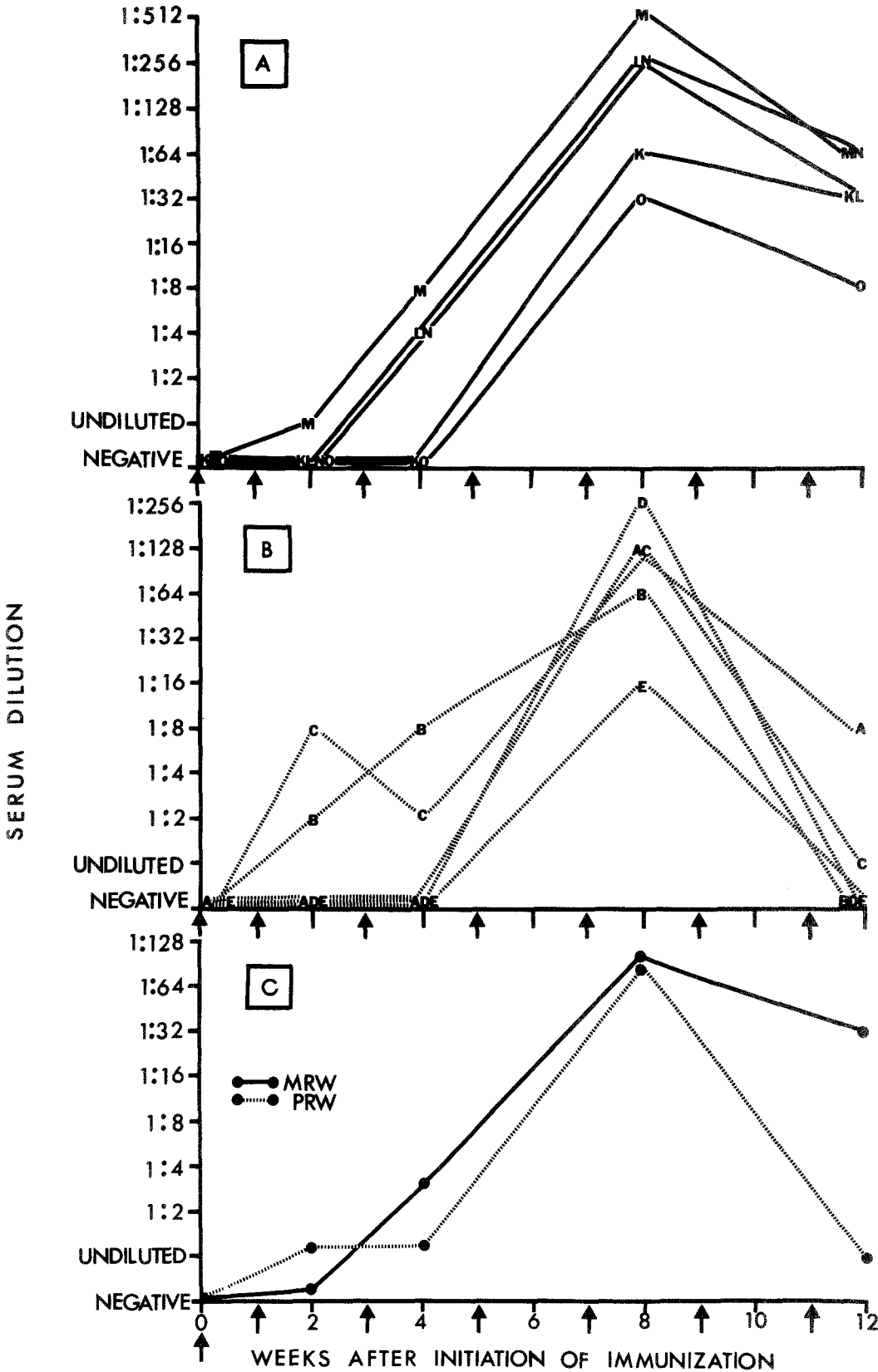


FIG. 4. A, Seventy-two-hour passive cutaneous transfer titers with the use of sera from rabbits immunized with MRW. B, Rabbits immunized with PRW. C, Means of titers of both groups of rabbits. Arrows indicate immunizations; letters denote individual rabbits.

TABLE II. Polystyrene tube radioimmunoassay demonstrating IgG antibody activity against monomer ragweed (MRW) and polymerized ragweed (PRW) and showing that both MRW and PRW contain exposed antigenic determinants of antigen E (AgE)

Polystyrene tube coating	Serum added	Isotope-labeled Ag added	Counts per minute bound
MRW	Human anti-RW	¹²⁵ I-MRW	2,003
	Normal	¹²⁵ I-MRW	303
PRW	Human anti-RW	¹²⁵ I-MRW	2,789
	Normal	¹²⁵ I-MRW	431
MRW	Human anti-RW	¹²⁵ I-AgE	4,448
	Normal	¹²⁵ I-AgE	49
PRW	Human anti-RW	¹²⁵ I-AgE	10,322
	Normal	¹²⁵ I-AgE	69

MRW, monomer ragweed; PRW, polymerized ragweed; AgE, ragweed antigen E.

Human anti-RW serum is from subject treated with standard RW immunotherapy.

Neutralization of human antibody activity against ragweed AgE by MRW and PRW

The maximal binding of ¹²⁵I-AgE by human anti-RW with the use of the ammonium sulfate precipitation technique was determined. Increasing concentrations of MRW and PRW were preincubated with the human anti-RW prior to addition of the ¹²⁵I-AgE (5 ng). The concentration of MRW and PRW required for 50% inhibition of binding of ¹²⁵I-AgE by anti-RW was determined. For MRW this was 118 ng and for PRW 386 ng. This experiment confirmed the presence of AgE determinants on MRW and PRW and demonstrated that approximately 3 times as much PRW was required as MRW for the same degree of neutralization of antibody against AgE.

DISCUSSION

We believe the results reported complete the research objectives outlined in the introduction to this paper. The studies demonstrated that a mixture of all ragweed proteins prepared by precipitation with ammonium sulfate and excluded by Sephadex G-15 can be polymerized by glutaraldehyde. The proteins in such a preparation have been identified as E(3), K(10), Ra-3 (11), BPA-R (12), and Ra-5.¹³ The smallest of these is Ra-5 which has a molecular weight of about 5,000 daltons, and thus we assume that most of the identified ragweed allergens should be included in MRW and PRW under the conditions of the preparations of our procedures of crude extract which are

similar to those described by Underdown and Goodfriend.¹¹

In studies of the immune response to MRW and PRW in rabbits, antibody against AgE was measured. This was done because every clinically useful modality of immunotherapy in humans should result in a response against major allergens in ragweed pollen and AgE is a major allergen of RW.³ The results of the studies indicated that PRW induced a greater immune response against AgE than did MRW.

The reagenic response against AgE in rabbits to immunization with MRW and PRW as demonstrated by the passive cutaneous transfer studies showed a less persistent reagenic response to AgE following PRW. This difference was not demonstrable in the direct cutaneous titers with AgE possibly because of the longer half-life of homocytotropic antibody bound to skin mast cells as compared with its half life in serum. Also direct skin titers will detect both IgE and IgG homocytotropic antibody while 72-hr passive transfer measures only IgE antibody.

PRW had 100-to 1,000-fold less allergenicity than MRW as demonstrated by reduced skin reactivity in human subjects.

Both MRW and PRW have antigenic determinants of AgE as demonstrated by immunization of rabbits, radioimmunoassay, and neutralization of human antibody against AgE. The latter experiment demonstrated that approximately 1/3 the amount of MRW as compared with PRW was required to neutralize antibody against AgE. We propose that the greater immunogenicity of PRW for AgE in rabbits at the same time that there are fewer exposed AgE determinants may be explained by sterically concealed antigenic determinants as previously suggested for polymerized antigens.¹⁴ The much greater reduction in allergenicity of PRW as compared with MRW, we believe, could be explained by the reduced number of exposed antigenic determinants of PRW, the decreased ability of high molecular weight polymers to diffuse to and bridge IgE antibody on mast cells in skin, and the lower molar concentration of PRW per unit weight. The ability of PRW to mount an enhanced immune response against allergenic determinants as demonstrated by the rabbit immune response against AgE may be explained by enhanced processing of the higher molecular weight polymers and their longer persistence in vivo. The concealed antigenic determinants in the polymerized preparation were apparently available to stimulate the humoral immune response.

The PRW preparation described here differs from the 2 polymerized AgE preparations previously de-

scribed^{1, 2, 4} which had molecular weight ranges of 200,000 to 4,000,000 and 4,000,000 to 20,000,000, respectively. No superiority in immunogenicity of either polymeric AgE to monomeric AgE was demonstrated in rabbits.^{1, 2} The increased immunogenicity reported with PRW in this study is not readily explained. PRW does have a wider molecular weight range (200,000 to 20,000,000) and is a complex copolymer of several monomeric species which could conceivably confer adjuvant properties.

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