

Studies on allergoids from naturally occurring allergens

III. Preparation of ragweed pollen allergoids by aldehyde modification in two steps

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We have devised a new process for modifying heat-labile allergens, which employs a sequential "two-step" incubation at temperatures of 10° C and 30° to 32° C. This process was found to produce effective ragweed "allergoids" with low allergenicity and good immunogenicity, which makes them useful for the therapy of allergic humans. Modification with formaldehyde produced derivatives ("formallergoids") that were about 10-fold less allergenic in allergic humans (as measured by leukocyte histamine-release assay), and similarly or more immunogenic in guinea pigs, than glutaraldehyde-modified allergens ("glutarallergoids"). Further analysis by RAST inhibition showed that a ragweed formallergoid was sixfold less reactive than a glutarallergoid with a pool of human IgE antibodies. However, the formallergoid had retained the ability to induce a wide array of antibodies against native ragweed antigens, since rabbit anti-formallergoid serum was able to recognize at least 12 different ragweed antigens, including AgE. Gel-filtration experiments showed that both the formallergoid and glutarallergoid materials contained polymers having apparent molecular weights distributed around 260,000 and 230,000 daltons, respectively (approximate range 30,000 to 900,000 daltons). Our studies provide the immunochemical basis for the use of these allergoids in the therapy of allergic humans. (J ALLERGY CLIN IMMUNOL 68:449, 1981.)

In previous experiments^{1, 2} we have shown that both purified group I rye grass allergen and dialyzed mixed grass pollen extracts can be converted into "allergoids" by mild treatment with formaldehyde solution. We have defined an allergoid as an allergen derivative that has greatly reduced allergenic reactivity in allergic humans relative to the native allergen but has largely retained desirable immunizing properties of the allergen, including an ability to induce IgG antiallergen "blocking antibody" production in humans and ex-

perimental animals.¹ This definition is not limited to formaldehyde-modified products but extends to other types of chemically and/or physically modified allergens with similar immunologic properties.

In adapting the original formalinization processes^{1, 2} from grass to short ragweed pollen allergens, we were confronted with the problem that important ragweed allergens, including AgE, are markedly heat labile. Thus in preliminary experiments we found that modification of purified AgE with 0.06M formaldehyde at 32° C, by procedures analogous to those used for rye group I, resulted in an extensive loss of immunizing properties (Marsh and Lichtenstein, unpublished observation). Incorporation of the additive L-lysine (0.05M) into the incubation mixture (cf. ref. 1) resulted in some retention of immunizing properties but to a degree considered inadequate for use in the immunotherapy of allergic humans. Extension of these studies to dialyzed crude ragweed extract showed an almost complete loss of desirable immunizing properties when the allergen was treated with either 0.36M formaldehyde alone or formaldehyde plus L-lysine

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Abbreviations used

ARs:	Allergenicity ratios
AgE:	Antigen E
RIA:	Radioimmunoassay
RAST:	Radioallergosorbent test
IEP:	Immunoelectrophoresis
CIE:	Crossed immunoelectrophoresis
BBS:	Borate-buffered saline

according to procedures similar to those previously described for production of grass allergoids.²

We concluded that it was necessary to devise a procedure whereby AgE (and other labile ragweed allergens) would be *mildly* denatured, without extensive loss of immunizing potency. Furthermore, since some heat-labile allergens probably occur in many allergenic extracts, such a method might be suitable for most allergens and produce more efficacious products. These considerations led to the concept of a two-step incubation procedure using formaldehyde and/or glutaraldehyde. The first step, modification at low temperature, would result in a modest degree of intermolecular and intramolecular cross-linking, with an expected small reduction in allergenic activity. Most importantly, this would lead to retention of desirable immunizing potency by stabilizing the native molecular conformation of heat-labile antigens under conditions of minimal thermal denaturation and would allow further modification at a higher temperature. The cross-linked product from the first step would be stable toward thermal degradation at the second step, and further reduction of allergenic reactivity should be achievable, with minimal loss of immunizing potency.

The aim of the present study was to establish the applicability of two-step aldehyde modification for the production of improved allergoid products for the immunotherapy (hyposensitization) of allergic humans.

MATERIALS AND METHODS

Ragweed extract (preparation A)

Dialyzed ragweed allergen was prepared as follows: Dried short ragweed (*Ambrosia elatior*) pollen (100 gm) was obtained from Greer Laboratories, Lenoir, N. C. It was defatted at room temperature with dry, peroxide-free diethyl ether (8 × 1 L successive volumes) and was sequentially extracted with three volumes (500, 250, and 250 ml) of 0.125M NH_4HCO_3 for 18, 4, and 4 hr at 4° C with gentle mechanical shaking. The supernatants were removed by centrifugation after each extraction and were subsequently combined. The combined extract was dialyzed extensively at 4° C in Visking size 18 cellulose casing against 0.002M

NH_4HCO_3 (5 × 72 L) and finally against distilled water (1 × 72 L) over a total period of about 4 days. The final dialysis was limited to about 5 hr to minimize precipitation of ragweed components. The dialyzed extract was centrifuged to remove a trace amount of precipitate, lyophilized, and stored in a sealed container at -20° C until used. It will be referred to as Prep. A. Other reagents utilized were formaldehyde (Fisher Scientific Co., Fair Lawn, N.J., certified ACS grade, 40% w/w) and glutaraldehyde (Eastman Kodak, Rochester, N. Y.: 50% w/w). All inorganic chemicals were reagent grade.

Formaldehyde modification (preparations B to E)

All the experiments involved sequential incubation steps at $10^\circ \pm 1^\circ \text{C}$ and $32^\circ \pm 1^\circ \text{C}$ with 0.1M Na_2HPO_4 - NaH_2PO_4 (NaP buffer) as the buffering agent, adjusted to pH 7.50 ± 0.05 at the beginning of each incubation. Details of reactant concentrations are given in Table I.

In brief, the procedures were as follows: Formaldehyde solution was slowly added to a solution of dialyzed ragweed extract (Prep. A) in NaP buffer at 5° to 10° C, with constant magnetic stirring and monitoring of the temperature and pH. Measured volumes of 2N NaOH were slowly added, as necessary, to maintain a pH range of 7.4 to 7.6. The final concentrations of the reactants were adjusted to those indicated in Table I by addition of NaP buffer and the final pH to 7.50 ± 0.05 by addition of 2N NaOH or 2N HCl. After the first incubation, the pH of the solutions had fallen by 0.03 to 0.09 units. After any adjustment of the formaldehyde concentration (Table I), the solutions were adjusted to pH 7.50 ± 0.05 prior to the second incubation. After the second incubation, solutions were dialyzed at 4° C against at least three 100-fold larger volumes of physiologic phosphate-buffered saline to remove excess aldehyde. Any volume changes during dialysis were noted in computing the concentrations of pollen solids. In certain cases the solutions were subsequently dialyzed extensively against distilled water at 4° C and were then lyophilized.

Glutaraldehyde modification (preparation F)

The experimental approach used for glutaraldehyde modification was similar to formaldehyde modification, except that incubation periods were much shorter because of the more rapid reaction of glutaraldehyde with proteins. Glutaraldehyde concentrations ranging between 0.0005M and 0.1M were investigated, but in preliminary experiments the derivative prepared with 0.025M glutaraldehyde (Table I) appeared to give the best results in terms of low allergenic activity and retention of desired immunizing properties. Detailed investigation of glutaraldehyde-modified pollen was therefore limited to investigation of just one derivative.

Allergenic activity

The allergenic activities of the four formalinized ragweed preparations (B, C, D, and E) and the glutaraldehyde-modified Prep. F were compared with that of the dialyzed ragweed allergen (Prep. A) from which they were derived

TABLE I. Conditions for two-step aldehyde modification of dialyzed ragweed pollen extract

Preparation	Modifying agent	Pollen (mg/ml)		Aldehyde (molar)		Time and temperature* of reactions	
		Step 1	Step 2	Step 1	Step 2	Step 1	Step 2
A	None	Not incubated					
B	Formaldehyde	2.0	2.0	2.0	0.36†	16 days (10°)	16 days (32°)
C	Formaldehyde	2.0	2.0	0.50	0.5‡	16 days (10°)	16 days (32°)
D	Formaldehyde	2.0	2.0	0.50	0.5‡	32 days (10°)	32 days (32°)
E	Formaldehyde	10	2.5	2.0	0.5‡§	16 days (10°)	16 days (32°)
F	Glutaraldehyde	2.0	2.0	0.025	0.025‡	20 hr (10°)	24 hr (30°)

*Temperatures were maintained to within $\pm 1^\circ\text{C}$.

†The solution was dialyzed against three changes of NaP buffer between steps 1 and 2 and fresh formaldehyde was then added.

‡The cited concentrations do not allow for the small uptake of aldehyde by pollen components in step 1.

§Before step 2, the solution was appropriately diluted with NaP buffer.

by means of the leukocyte histamine-release assay of Lichtenstein and Osler³ as modified by May et al.⁴ ARs for 15 different highly ragweed-sensitive patients were calculated from the concentrations of modified allergen, relative to native allergen, which elicited 50% histamine release from the patients' cells.¹

Immunogenicity by inhibition of leukocyte histamine release

The relative immunogenic properties of the native and aldehyde-modified allergens (except Prep. C) were investigated by their capacities to induce antiallergen antibody production in guinea pigs. Antigen-alum adsorbates were prepared by mixing each antigen (2.0 mg/ml) with an equal volume of alumina gel suspension (50 mg/ml), prepared according to the Willstaetter procedure.⁵ The conjugates were allowed to stand at 4°C for 24 hr prior to injection to facilitate optimal adsorption of the antigens to the alum. Optical density measurements on the supernatants of centrifuged antigen-alum mixtures and the original antigen solutions indicated greater than 90% adsorption of the antigens had occurred.

Under controlled conditions, groups of 11 random-bred Hartley strain guinea pigs (350 to 550 gm), matched for age, weight, and sex, were immunized intramuscularly with the antigen-alum conjugates in two sites ($2 \times 1.0\text{ ml}$) in the hind legs. This procedure was repeated 21 days later, and injections of half these quantities of antigen and adjuvant were given at 34 days in a similar manner. The animals received two further sets of boosters of aqueous antigen (100 and 160 μg) administered intradermally in the back (at four sites, 25 or 40 μg per site) at 41 and 48 days, respectively. The animals were exsanguinated nine days later and the serum was collected.*

The antiallergen antibody contents of the five serum pools (AA, AB, etc.) were determined by their ability to

inhibit allergen-mediated histamine release from the leukocytes of three human ragweed-allergic donors.¹ In brief, leukocytes were incubated in vitro with guinea pig antiserum (diluted 1/700) and ragweed allergen (at a concentration that in the absence of serum, would release approximately 80% of the leukocyte histamine). The inhibition of histamine release resulting from the presence of the antiserum was computed by comparing the release in appropriate controls containing leukocytes, antigen, and normal guinea pig serum (1/700). The choice of 1/700 guinea pig serum for this experiment was made by a preliminary titration, which showed that this dilution provided inhibition values falling primarily on the linear portions of the inhibition curves (i.e., in the range of about 10% to 90% inhibition) for most serum-leukocyte combinations.

Immunogenicity by RIA

Guinea pig antibody responses toward Preps. A, B, D, E, and F were further investigated by RIA employing highly purified ragweed AgE⁶ labeled with ^{125}I . The assay was similar to that described elsewhere^{7, 8} for the measurement of human IgG responses. Briefly, individual sera or serum pools were diluted to 1/40 in BBS at pH 8.3; further dilutions were made in guinea pig IgG in BBS (50 $\mu\text{g N/ml}$). One hundred microliters of serum at dilutions of between 1/100 and 1/100,000 were incubated with ^{125}I -AgE (100 μl containing 5 ng immunoprecipitable AgE/ml) for 5 hr at 23°C . The AgE-antibody complexes and carrier IgG were precipitated by addition of a slightly greater than equivalent amount of goat anti-guinea pig IgG, followed by incubation overnight at 4°C . The precipitates were washed with BBS ($4 \times 3\text{ ml}$) and counted in a gamma counter, and the antibody contents (in arbitrary units/ml, U/ml) were computed by reference to a standard pool (pool Q) from guinea pigs previously immunized with crude ragweed allergen.

RAST inhibition

The allergenic activities of formaldehyde-modified Prep. E and glutaraldehyde-modified Prep. F, in comparison with the allergen (Prep. A), were further examined by RAST-inhibition experiments.

*Two guinea pigs died during the experiment because of infection, and one serum sample was lost during processing; hence the numbers of remaining sera are less than 11 in certain groups.

TABLE II. Allergenicity ratios (ARs) of modified ragweed antigens relative to dialyzed native ragweed by leukocyte histamine-release assay

Cell donor	Cell sensitivity to dialyzed ragweed extract, Prep. A ($\mu\text{g/ml}$ for 50% histamine release)	AR				
		Prep. B	Prep. C	Prep. D	Prep. E	Prep. F
1†	2.7×10^{-4}	300	200	278	455	ND
2	4.0×10^{-4}	ND	ND	ND	1667	ND
3	8.5×10^{-4}	454	769	ND	ND	ND
4†	8.8×10^{-4}	625	263	625	909	ND
5†	1.2×10^{-3}	4166	3125	10,000	4350	ND
6†	2.0×10^{-3}	400	154	456	1300	ND
7†	2.4×10^{-3}	278	120	500	370	ND
8†	2.8×10^{-3}	118	50	156	179	ND
9†	3.6×10^{-3}	2173	769	2040	5556	ND
10†	9.0×10^{-3}	222	100	476	385	ND
11	9.5×10^{-3}	179	208	ND	ND	ND
12†	1.4×10^{-2}	454	150	417	556	ND
13‡	4.2×10^{-4}	490	ND	340	1050	73
14‡	7.0×10^{-4}	200	ND	430	715	74
15‡	1.0×10^{-3}	90	ND	130	200	25
Geometric mean ratios						
All tested patients		386	254	529	789	51
Tested with Preps. B to E (n = 9)		508	229	664	829	—
Tested with Preps. B and D to F (n = 3)		207	—	267	531	51

$$\text{AR} = \frac{\text{concentration of modified allergen eliciting 50\% histamine release}}{\text{concentration of native allergen eliciting 50\% histamine release}}$$

(This ratio represents the number of times the concentration of modified allergen has to be increased so that it may induce the same degree of histamine release from the leukocytes of allergic people as the native allergen from which it was derived. In each case, the antigen concentrations are expressed in terms of micrograms of nondialyzable pollen solids per milliliter).

ND = not determined.

†Patients studied with preparations B to E.

‡Patients studied with preparations B and D to F.

Ragweed-coupled beads were prepared as follows. Cyanogen bromide-activated Sepharose 4B (Pharmacia) was allowed to react with Prep. A (250 mg/10 gm of beads) in 50 ml of "coupling buffer" (0.1M NaHCO₃, 0.5M NaCl at pH 8.4) for 18 hr at 4° C with end-over-end rotation of the reaction mixture. The beads were centrifuged, washed with 3 × 25 ml coupling buffer, incubated for 2 hr at 23° C (with rotation) with 1M ethanolamine adjusted to pH 7.5, and finally washed with six changes of "wash buffer" (0.5 gm of NaH₂PO₄, 13.4 gm of Na₂HPO₄ · 7H₂O, 22.3 gm of NaCl, and 0.5 gm NaN₃, adjusted to 1 L with H₂O; pH 7.5). The recommended washing with pH 4 buffer was not utilized because of the instability of AgE at this pH.⁹ Optical density measurements (at 280 nm) of the solution of Prep. A and the supernatant after the initial coupling reaction indicated that 68% of the allergen had been coupled.

The methodologic approach for the RAST-inhibition experiments was similar to that previously described by Gleich et al.¹⁰ using ragweed-cellulose beads. A pool of 10 sera was prepared from nonimmunized highly ragweed-sensitive subjects who were known to have high anti-ragweed IgE titers. ¹²⁵I-anti-IgE was prepared from immunoadsorbent-purified, ϵ -specific rabbit antibody.¹¹ Preliminary titration experiments, in which the concentrations of the serum pool and the ragweed beads were varied, showed that 0.1 ml of a

1/10 dilution of the allergic serum pool with 0.5 ml of 0.1% v/v of packed ragweed-beads in RAST buffer (containing 4.2 ml/L Tween 20 and 2 gm/L bovine serum albumin) were optimal for the RAST-inhibition assay. The abilities of the allergen (Prep. A) and modified allergens E and F to inhibit binding of the serum IgE antibody to the ragweed-beads were investigated by incorporating 0.1-ml volumes of a series of different concentrations of the antigens into the mixtures of ragweed-beads and diluted allergic serum. Buffer was substituted for the antigen in positive control tubes; negative controls contained ragweed-beads plus 1/10 serum from a nonallergic subject. All mixtures were allowed to incubate for 18 hr at 23° C, with continual mixing. The ragweed-beads were then washed with 3 × 3 ml RAST buffer, reincubated overnight with ¹²⁵I-anti-IgE, rewashed, and counted. Five percent bovine serum albumin was used in the RAST buffer for the second incubation to reduce nonspecific adsorption of ¹²⁵I-anti-IgE to 0.1% to 0.2% of the total added counts per minute. All experiments were performed in triplicate. The mean percent inhibition was computed relative to a positive control containing no inhibitor (after appropriate subtraction of the negative control). Allergenicity ratios were computed for the modified allergens based on the concentrations of modified allergen and native allergen inducing 50% inhibition of RAST binding.

Immunodiffusion and immunoelectrophoresis

Rabbit antisera were prepared by immunizing a group of rabbits intramuscularly with 10 mg of formalinized antigen (Prep. C, one rabbit; Prep. E, two rabbits) emulsified with complete Freund's adjuvant. Six subsequent courses of 2 mg of antigen emulsion were administered every week, and the animals were subsequently boosted with 100 μ g of antigen administered intradermally as an aqueous solution. Serum samples were taken 7 to 10 days after the sixth and subsequent immunizations. The IgG fraction from the serum pool was obtained as described elsewhere.¹² Rabbit anti-ragweed IgG antibodies (anti-Am.e.76),¹³ rabbit anti-AgE,¹⁴ goat anti-Ra3,¹⁵ and goat anti-Ra5¹⁶ were the same as those previously described. Highly purified AgE, Ra3, and Ra5 were similar preparations to those described elsewhere.⁷⁻¹⁴ Analysis by immunodiffusion, IEP, and CIE of the allergen and formalinized allergen was made with all of these sera by methods similar to those previously described.¹³⁻¹⁶

Molecular weight distribution

The degrees of polymerization of formaldehyde- and glutaraldehyde-modified materials (Preps. E and F) relative to the allergen (Prep. A) were compared by chromatography on Sephadex G100 Superfine (150 cm by 1.5 cm diameter) and Sepharose CL-4B (130 cm by 1.5 cm diameter) columns in 0.2M ammonium bicarbonate buffer. Each antigen solution (4 to 8 mg in 2.0 ml) was applied to the columns, which were run at constant rates of about 7.5 ml/hr by means of a peristaltic pump. Fractions were collected every 30 or 60 min and were accurately measured for each experiment with a Hamilton microsyringe to determine the precise flow rates. The O.D._{280nm} profiles of the eluted peaks were automatically recorded with an ISCO UA5 recorder. The Sephadex column (molecular weight fractionation range approximately 4000 to 100,000 daltons) was calibrated with 2.0-ml samples of mixtures of the following marker proteins: horse cytochrome-c (12,400 daltons), bovine chymotrypsinogen A (24,500 daltons), ovalbumin (43,000 daltons), human serum albumin (68,000 daltons), and horse ferritin (440,000 daltons), and the apparent molecular weights of the O.D. peaks of eluted materials were determined from plots of elution volume vs log[mol. wt.]. The Sepharose column (fractionation range approximately 60,000 to 20 million daltons) was similarly calibrated with ovalbumin, albumin, ferritin, rabbit aldolase (158,000 daltons), bovine thyroglobulin (669,000 daltons), and blue dextran (2,000,000 daltons). The elution volumes for AgE in the allergen elution profiles were determined by immunodiffusion analysis with anti-AgE.

RESULTS

Allergenic activity

Table II shows results of 15 sets of histamine-release assays comparing the allergenic activities of various formalinized allergens (Preps. B to E) and glutaraldehyde-modified allergen (Prep. F) relative to the unmodified allergen (Prep. A). The AR values

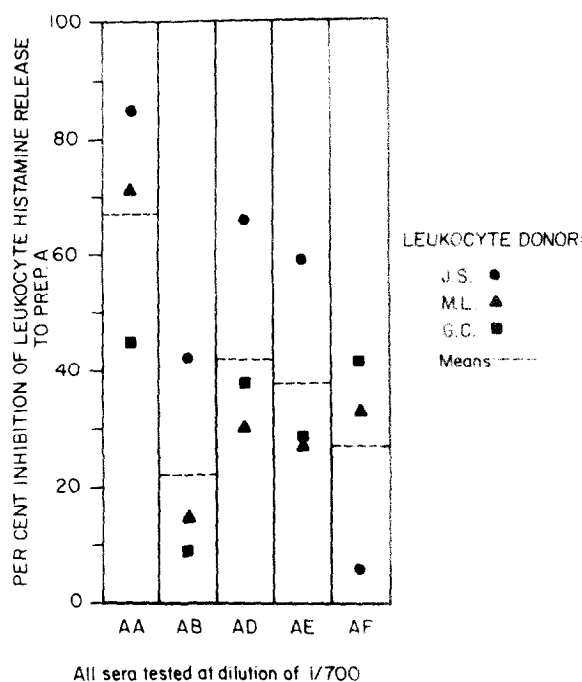


FIG. 1. Inhibition of leukocyte histamine release to ragweed allergen (Prep. A) by means of guinea pig antisera. AA, AB, etc. were pools of 10 to 11 sera raised against antigen preparations A, B, etc. The greater the degree of inhibition of allergen-induced histamine release, the greater the blocking antibody content of the serum pool.

varied both between preparations and between allergic subjects, depending on the degree of modification of the various allergenic determinants and the specific determinants recognized by a particular individual's IgE antibodies. Since the same preparations were used in all cases, the probable reason for the wide variation in AR values between individuals is that different individuals recognize different determinants on the various ragweed allergen molecules. It is presumed that these determinants have been altered to various degrees in a given modified allergen preparation. Hence the degree of reduction in allergenicity would be expected to vary from person to person. Similar results were observed previously with formalinized rye group I and mixed grass preparations.^{1, 2}

Of the formalinized products, Prep. E was the least and Prep. C the most allergenically active according to geometric mean AR, with Prep. E about 800-fold and Prep. C about 200-fold less allergenic than the native allergen in the nine highly ragweed-sensitive subjects tested with Preps. B to E (see footnote to Table II for identification of patients). By two-tailed paired *t* tests, the significance of the differences in log AR between Preps. E and D, Preps. D and B, and Preps. B and C were, respectively, $0.1 > p > 0.05$; $0.05 > p > 0.025$; $p < 0.005$. As we previously

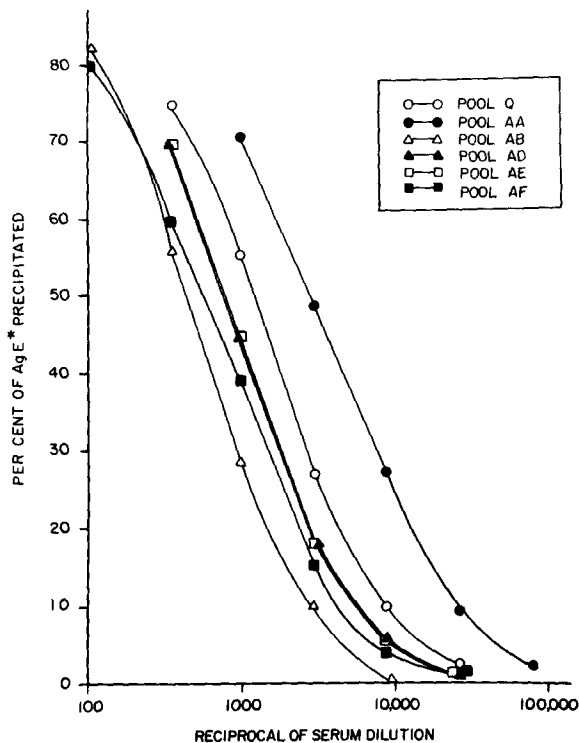


FIG. 2. Binding curves showing the relative abilities of pooled guinea pig sera AA, AB, etc. (raised against Preps. A, B, etc.) to bind to radiolabeled ragweed AgE. Pool Q is a standard serum pool raised against a different ragweed allergen preparation, which was run as a standard.

found for formalized rye grass group I,¹ the AR values were not related to the patient's response to the allergen. In further tests, we found that the allergenic activities of the two-step formalized materials were 10-to-100-fold less than those after the first incubation step.

Comparison of the allergenic activities of formalized Preps. B, D, and E with glutaraldehyde-modified Prep. F in three subjects (Nos. 13 to 15, Table II) revealed that the formalized preparations were all substantially less allergenic than Prep. F, with Prep. E having a 10-fold lower geometric mean AR than that of Prep. F ($p < 0.01$ by paired t test).

Immunogenicity by inhibition of histamine release

The capacities of five guinea pig serum pools (AA, AB, AD, AE, and AF) to inhibit allergen-mediated histamine release are shown in Fig. 1 for assays utilizing three different patients' leukocytes. Comparison of the mean inhibition values for each serum pool (diluted 1/700) from the three donors showed that serum pools AB, AD, AE, and AF were, respectively, 33%, 66%, 57%, and 40% as effective in in-

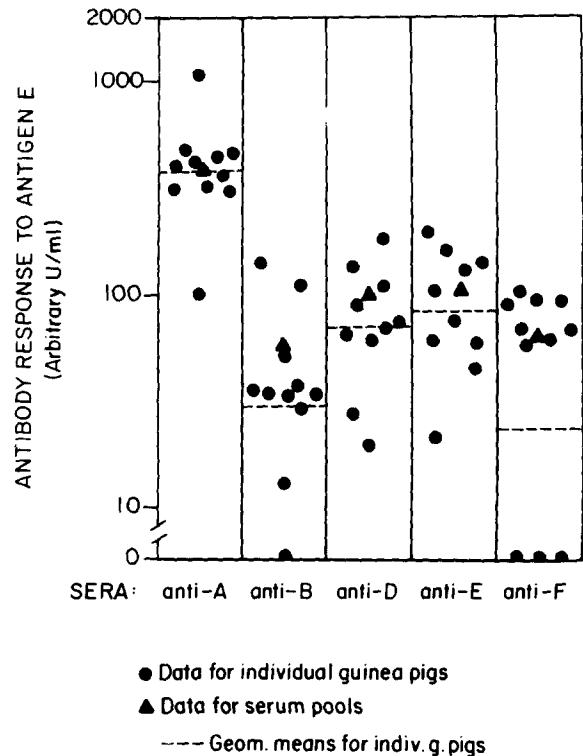


FIG. 3. Investigation of the individual guinea pig antisera in terms of their ability to bind radiolabeled ragweed AgE. Geometric mean values and values for pooled sera are shown for comparison purposes.

hibiting histamine release as the anti-allergen pool AA. Probably because of the small numbers of people tested, the differences between the serum pools were not significant by paired t test, except for the comparison of pool AA vs AB ($p < 0.02$). There appear to be donor-to-donor differences in the relative degrees of inhibition observed in testing antisera raised toward formalized vs glutaraldehyde-modified allergens (compare, particularly, the data for donor J. S. vs those for G. C.). Overall, these results show that guinea pigs immunized with the various aldehyde-modified preparations, particularly formalized Preps. D and E, made appreciable amounts of cross-reactive "blocking" antibody against allergen Prep. A.

Immunogenicity by RIA

Titration of the serum pools by RIA (Fig. 2) showed that the anti-allergen pool AA had the highest amount of anti-AgE IgG antibody. The two best anti-modified allergen pools were, again, AD and AE, each of which possessed 28% of the antibody of pool AA, determined at the serum concentrations inducing 50% precipitation of ^{125}I -AgE. (Slightly different values were obtained with other AgE precipita-

tion values, since the AgE-binding curves were not exactly parallel.)

The anti-AgE IgG levels of the individual guinea pig sera were tested at concentrations giving AgE-binding values falling on the linear portion of the binding curve for the standard serum (pool Q). Antibody contents, computed for the undiluted individual sera and serum pools, are shown in Fig. 3. The geometric means for the individual sera within each pool vs the values for the respective pools sometimes differed markedly (as expected), especially in the case of anti-F vs. AF, where three individual sera contained no detectable antibody. According to geometric means, anti-D and anti-E sera were more potent than anti-B and anti-F, their respective potencies being 19% and 22% vs 8% and 6% of anti-A. By two-tailed t tests on log antibody values of individual sera, all groups of anti-modified allergen sera were significantly less potent than anti-A ($p < 0.001$); anti-E and anti-D were not significantly different; and anti-B were significantly less than anti-E ($p < 0.05$); anti-F were less than anti-E ($p < 0.1$).

The above studies suggest that Prep. E possessed the optimal properties desired in an allergoid, i.e., very low allergenicity with good retention of desirable immunizing properties. Subsequent experiments were designed primarily to compare Preps. A and E, although Prep. F was included in certain experiments to provide further comparison with a glutaraldehyde-modified product.

RAST inhibition

The relative IgE-binding activities of Preps. A, E, and F were studied by RAST inhibition. The titration curves shown in Fig. 4 compare the relative abilities of the three antigens to inhibit binding of a heterogeneous pool of anti-ragweed IgE antibodies to ragweed-beads. The slopes of the inhibition curves for the two modified allergens and for the native allergen were not significantly different. Allergenicity ratios computed at the points of 50% inhibition were 3010 in the case of Prep. E and 470 for Prep. F.* These data reflect a 6.4-fold lower allergenicity of Prep. E relative to Prep. F.

Immunodiffusion and IEP experiments showed that all the modified antigens had largely lost their ability to react with hyperimmune rabbit anti-whole ragweed sera. AgE, Ra3, and Ra5 were undetectable in Preps.

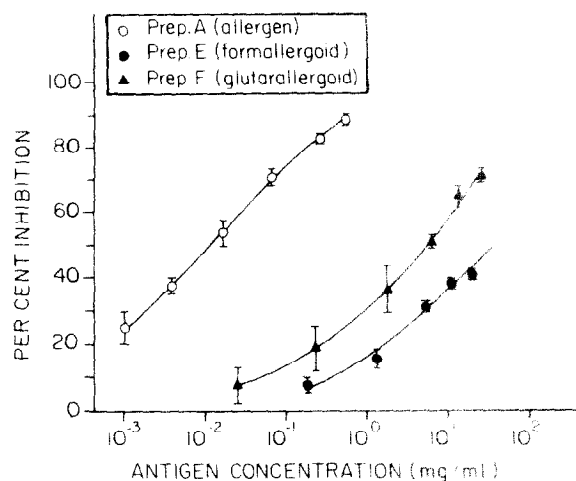


FIG. 4. RAST-inhibition experiments comparing the abilities of Preps. A, E, and F to inhibit binding of human anti-ragweed IgE antibodies to ragweed-beads. Concentrations are expressed in milligrams of nondialyzable pollen antigen per milliliter. Bars, Standard deviations of the means.

E and F by immunodiffusion against rabbit anti-crude ragweed or anti-AgE, anti-Ra3, and anti-Ra5. On the other hand, rabbit anti-formalinized antigen (anti-formallergoid) reacted strongly with both the native antigen (Prep. A) and formalinized antigen (Prep. E). With this antibody in CIE experiments, about 12 precipitates were detectable in Prep. A and about four in Prep. E (Fig. 5, A and B). In IEP experiments, this antibody reacted with AgE (Fig. 5, C) but not Ra3 and Ra5.

Molecular size fractionation

The chromatographic elution O.D. profiles were recomputed to reflect readings for 8-mg column loads of Preps. E and F and 32 mg of Prep. A (Fig. 6). The Sephadex G100 chromatogram showed that polymerization had occurred in Preps. E and F as a result of aldehyde treatment. Both materials were eluted as single peaks at the exclusion volume of the column indicated by the ferritin marker (Fig. 6, A). The elution profiles showed some trailing toward the lower molecular weight range, apparently because of adsorption to Sephadex, since similar trailing was not observed in Sepharose chromatography. The chromatogram of Prep. F also showed a peak of low molecular weight material, probably including glutaraldehyde, since the routine dialysis step had been omitted for this particular sample. Allergen Prep. A contained some high molecular weight material eluted at the void volume of the G100 column, as well as pigmented low molecular weight substances that were greatly retarded, presumably the result of adsorption to the Sephadex. An-

*Note that the curve for Prep. F was extrapolated to 50% inhibition. Higher concentrations of Preps. E and F (≥ 500 mg/ml added to the incubation mixture) needed for greater inhibition were not available; therefore, the complete inhibition curves could not be plotted.

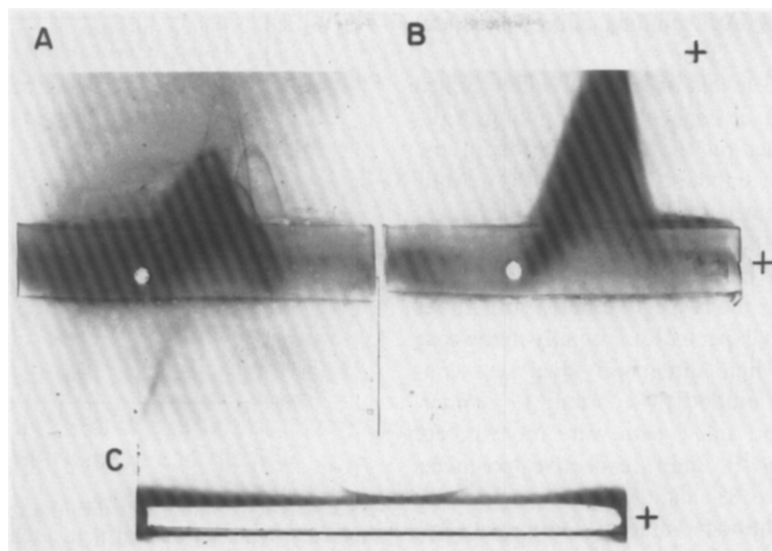


FIG. 5. **A** and **B**, CIE experiments with allergen Prep. **A** (150 μ g nondialyzable pollen solids) in **A** and formallergoid Prep. **E** (150 μ g nondialyzable pollen solids) in **B** against rabbit anti-formallergoid antibodies (500 μ l in the acidic gels and 200 μ l in the basic gels). The first-dimension gels were run for 30 min at 10 V/cm and the second-dimension gels for 18 hr at 2 V/cm. Anodes are to the right (first dimension) and top (second dimension). **C**, IEP of AgE (5 μ g) against rabbit anti-formallergoid. Anode is to the right.

tigenic material of molecular weight ca. 5000-70,000 daltons was separated on the column, with the maximum for AgE being at an apparent molecular weight of $30,000 \pm 2000$ daltons. A more accurate elution volume for AgE, obtained with a purified AgE preparation, corresponded to a molecular weight of $33,000 \pm 1000$ daltons. This is the same value as that obtained in other gel-filtration experiments.¹³ Since this estimate is lower than the values of 37,000 and 38,000 daltons previously determined by other methods,⁹ it is possible that AgE is retarded by adsorption to the Sephadex.

A Sepharose CL-4B column was used to resolve the higher molecular weight aggregates of the aldehyde-modified materials (Fig. 6, *B*). The elution profile for Prep. E showed a single slightly asymmetric peak, the maximum of which eluted at an apparent molecular weight of $260,000 \pm 10,000$ daltons. The peak was, however, about three times broader than that of the aldolase marker. Comparison of the elution profile for Prep. E with the profiles of the marker proteins suggested a distribution of between 30,000 and 900,000 daltons for this preparation. The elution profile for Prep. F showed a complex O.D. peak with three maxima, the first at an apparent molecular weight of $230,000 \pm 20,000$ daltons (indistinguishable from that of Prep. E) and the latter two at less than 40,000 daltons. It was surprising to find these latter two peaks in this experiment because the material applied to this column had been extensively dialyzed against 2×10 L of buffer with the aim of

removing any residual low molecular weight components (especially glutaraldehyde).

DISCUSSION

Our studies have shown that treatment of dialyzed ragweed pollen extract with formaldehyde or glutaraldehyde with a new two-step process at a temperature of 10° C followed by 30° or 32° C leads to the production of allergen derivatives having the properties of "allergoids."¹¹ To distinguish between different types of allergoid, those prepared with formaldehyde and glutaraldehyde will be termed "formallergoids" and "glutarallergoids," respectively. The present studies suggest that modification with formaldehyde may be more effective than glutaraldehyde modification in producing allergoids that exhibit both very low allergenicity in allergic humans and minimal loss of immunogenicity (particularly IgG "blocking antibody" production)—characteristics desirable for the therapy of allergic humans. However, further studies with different reaction conditions would be needed to confirm this possibility (see also, discussion of molecular weight-fractionated derivatives below). All of the formallergoids studied here were less allergenic and either similarly or more immunogenic than the glutarallergoid. The best preparations (*D* and *E*) were 600 to 800-fold less allergenic by leukocyte histamine-release assay (Table II) and were found by three methods (Figs. 1 to 3) to retain about 20% to 60% of the desirable immunizing properties of the

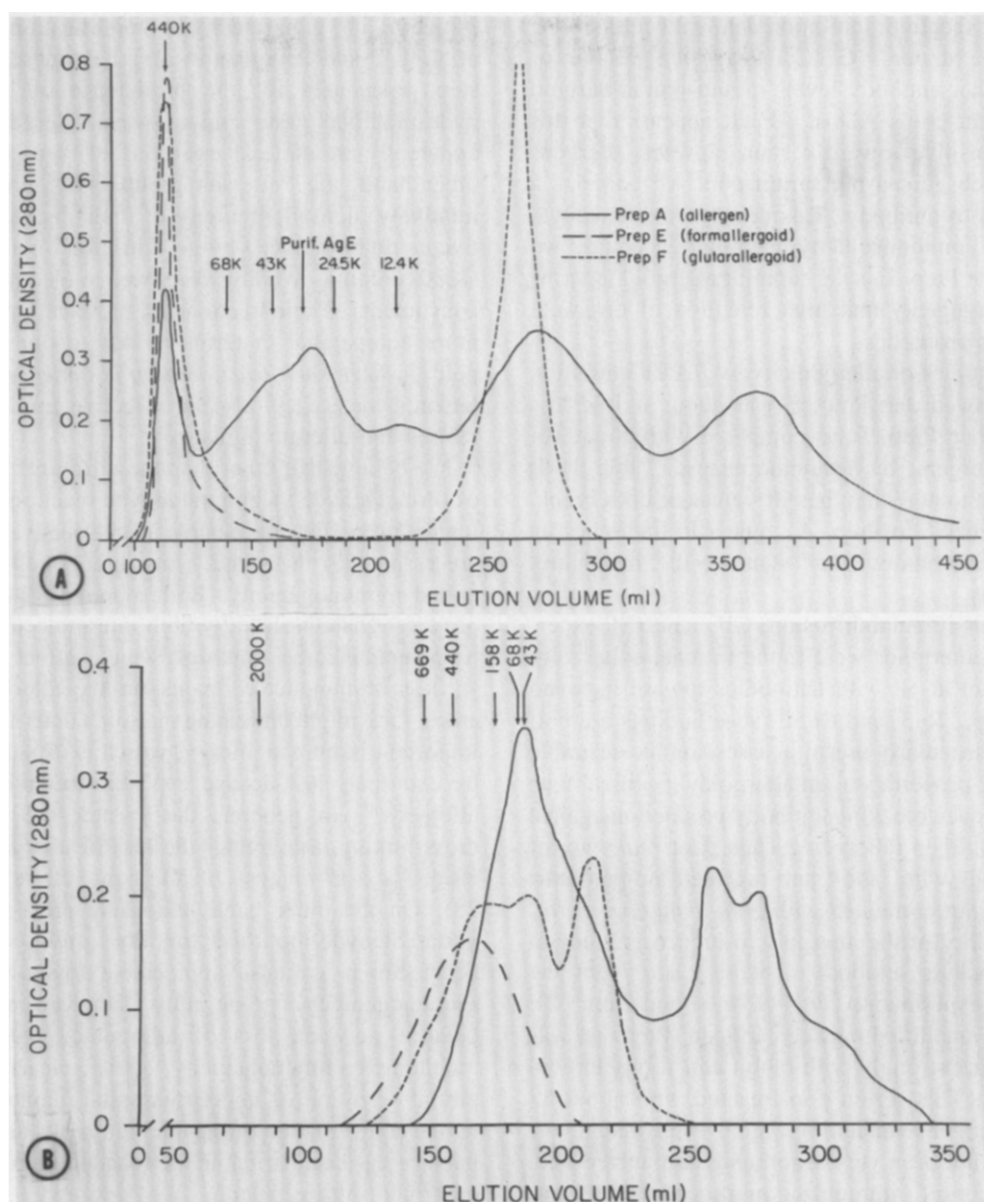


FIG. 6. Gel-filtration of 2-ml samples of Preps. A, E, and F on columns of: **A**, Sephadex G100, Superfine (150 cm by 1.5 cm diameter) and **B**, Sephadex CL-4B (130 cm by 1.5 cm diameter). The elution profiles are equivalent to 8 mg each of Preps. E and F and 32 mg of Prep. A. (The profiles for Prep. A were adjusted to reflect a larger amount of antigen to show the peaks more clearly.) Arrows, Elution volumes for marker proteins (kilodaltons) and for AgE.

native allergen (Prep. A). Conversely, glutarallergoid Prep. F was only about 50-fold less allergenic than the native allergen and retained only about 5% to 40% of its immunizing properties. Thus the two-step process resulted in allergoids with various degrees of potential therapeutic utility depending on both the modifying agent and the reaction conditions.

Studies of blocking antibody production were carried out by two different procedures—inhibition of allergen-induced leukocyte histamine release (using

allergic human leukocytes as the assay system) and binding of ^{125}I -AgE, as investigated by double-antibody RIA. Not surprisingly, the different assays led to somewhat different estimates of the relative amounts of antibody activity in the anti-allergoid sera relative to anti-allergen sera. On the other hand, they showed that two formallergoids, Preps. D and E, were generally more effective than the other allergoids in eliciting blocking antibody production (Figs. 1 to 3). In the leukocyte assay (Fig. 1) there was one

interesting exception; inhibition data with cells of one of the three donors (G. C.) suggested similar or slightly higher antibody levels in anti-glutarallergoid pool AF than pools AD or AE. It appears that this donor is sensitive toward certain ragweed allergens toward which guinea pig serum pool AF contains a higher level of antibody. Taking all of the leukocyte assays and immunogenicity data into account, we conclude that Prep. E is the superior allergoid in terms of low allergenicity and high retention of desirable immunizing properties.

Further studies of allergenicity by RAST inhibition (Fig. 4) showed that Prep. E was about sixfold less allergenic than Prep. F, as compared with about 10-fold less allergenic by histamine release (Table II). It is of interest to note that the AR values of both Preps. E and F (relative to Prep. A) appear to be higher by the RAST-inhibition assay than by the histamine-release assay.

By various immunodiffusion, CIE, and IEP experiments, the allergoids were found to react either very poorly or not at all with antibodies toward ragweed antigens AgE, Ra3, and Ra5. These findings are not, however, particularly useful in assessing the desirable immunizing properties of an allergoid, specifically its ability to induce blocking antibody production against the native antigen (allergen). Thus poor *reactivity* of the allergoids with rabbit anti-ragweed antigen antibodies was not correlated with poor *immunogenicity*, i.e., the ability of the allergoid to induce production of IgG blocking antibody. This is clear from the guinea pig experiments (Figs. 1 to 3) and from CIE where the unmodified ragweed antigen (Prep. A) was found to react strongly with rabbit anti-formallergoid (Fig. 5, A). This serum pool reacted with about 12 ragweed antigens (including AgE), even though the rabbits had not been very vigorously immunized with the allergoid (cf. immunization procedures recommended in refs. 12 and 13.). Antigens Ra3 and Ra5 could not be detected with the rabbit antiformallergoid, which is not surprising, since these antigens are usually undetectable (or difficult to detect) with even the most potent rabbit anti-ragweed antigen sera.¹⁵ We would anticipate that even better IgG responses toward AgE and other ragweed antigens would be obtained when allergoids are administered to allergic humans who are *already* making IgG (as well as IgE) responses to selected ragweed antigens toward which they are sensitive.¹⁷⁻²⁰

It would appear that the two-step aldehyde-modification procedure minimizes undesirable denaturation, which represents a particular problem with ragweed. (This is especially true for the slower chemical modification with formaldehyde and may be less of a

problem in glutaraldehyde modification.) Moran et al.^{21, 22} and Patterson et al.²³ have described one-step procedures for the production of glutaraldehyde-modified grass and ragweed allergens, which apparently are quite allergenic (like Prep. F). On the other hand, the Patterson group^{23, 24} found that the relatively high allergenicity of their ragweed derivative could be reduced by gel filtration on a Sephadex G200 column to remove lower molecular weight constituents. Their fractionated product appears to be more comparable in terms of AR to our formallergoids, *where such fractionation is unnecessary*. Patterson's material is also an effective immunogen in rabbits and allergic humans.^{23, 25}

We believe that other than the different chemistries of formaldehyde vs glutaraldehyde reactions, the biological properties one desires in an effective allergoid¹ are essentially the same—low allergenicity with retained immunogenicity. Both aldehydes give rise to products characterized by both intermolecular and intramolecular cross-linking, which effect primarily surface immunodeterminants on the allergen molecules. One might anticipate that such determinants are important in human allergy, which is usually induced by extremely low-dosage exposure to environmental allergens.²⁶ In general, the greater the degree of cross-linking, the greater the loss of allergenic properties for a given type of allergoid (cf. refs. 22 and 25). On the other hand, extensive cross-linking of glutaraldehyde-modified timothy grass extract has been shown to cause appreciable loss of desirable immunogenicity.²⁵ One must therefore maintain a balance between loss of allergenic reactivity and maintained immunogenicity, which clearly requires partial retention of certain essential elements of the native conformation of the allergen molecules. Such a balance has been struck in the formallergoid materials described in this paper.

The gel-filtration results (Fig. 6) show that the main physicochemical differences between ragweed formallergoid and glutarallergoid lie in the presence of low molecular weight material in the glutarallergoid. The molecular size distributions of polymeric materials in both preparations are very similar, roughly 30,000 to 900,000 daltons, with the maxima in the elution profiles on Sepharose CL-4B being at apparent molecular weights of 260,000 and 230,000 daltons for the formallergoid and glutarallergoid, respectively (Fig. 6, B). The second and third maxima in the glutarallergoid profile contain low molecular weight pigmented components that were not removed by extensive dialysis. Patterson's^{23, 24} "polymerized ragweed" appears to have a somewhat higher molecular weight distribution than either the formallergoid or

glutarallergoid described here, although a detailed comparison of the product is needed to investigate this question further.

In conclusion, the present studies provide an immunologic basis for the use of two-step ragweed allergoids in the therapy of ragweed-sensitive subjects, discussed in the succeeding²⁷ and future papers. Much higher doses of allergoids can be administered because of their low allergenicity, and good protective immunologic responses ensue because desirable immunizing properties (including IgG blocking antibody production) have been retained.

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