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Studies on "allergoids" prepared from naturally occurring allergens

II. Evaluation of allergenicity and assay of antigenicity of formalinized mixed grass pollen extracts

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A crude dialyzed mixed grass pollen extract was modified under mild conditions with formaldehyde in the presence or absence of low molecular weight additives which participate in the chemical reaction. By scratch and intradermal skin testing of grass-sensitive subjects, certain of the derivatives (allergoids) were found to be about 200 to 2,000 times less allergenically reactive than the native pollen extract (allergen). The allergoids largely retained their capacity to induce guinea pigs to produce antibody which strongly cross-reacted with the allergen, as measured by passive cutaneous anaphylaxis (PCA) in normal guinea pigs. Cross-antigenicity between the allergen and allergoids was also observed by immunodiffusion analysis with the use of anti-allergen and anti-allergoid sera. One of the formalinized derivatives also was studied by bronchial inhalation challenge experiments in 5 asthmatic subjects.

At least three types of immunologic and cellular changes have been described as a result of immunotherapy of allergic human beings with the use of pollen allergens: (1) formation of "blocking antibodies,"^{1,2} largely of the

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IgG type³; (2) reduction in cellular responsiveness as assessed by the capacity of the basophilic leukocytes to release less histamine upon challenge by specific allergen or anti-IgE *in vitro*^{4-6, 10}; (3) long-term decrease in the mean titer of serum reaginic (IgE) antibody.⁷⁻¹⁰ None of these changes singly accounts for the clinical results achieved in immunotherapy, and no simple relationship has yet been established between the magnitude of these changes and the degree of symptomatic relief of the treated patients. Nevertheless, it has been shown on a statistical basis that hay fever patients receiving high doses of antigen (mean about 10,000 protein nitrogen units [PNU] or greater of crude ragweed extract per season) experienced much greater symptomatic relief than patients on low-dosage immunotherapy (mean about 1,000 PNU of crude ragweed extract).^{13*} Moreover, for pollens there is a significant correlation between the total antigen dose and the level of antigen-neutralizing or "blocking" antibody^{11, 12} and a somewhat less-precise relationship between the dose and the degree of reduction in cellular responsiveness.^{4, 10}

Thus, the available evidence points out the importance of the dosage achieved during the course of immunotherapy, as well as the limitations of conventional injection therapy with the use of native allergens in the commonly accepted but relatively low dose range. These low-dose regimens continue to be used in the general practice of allergy, largely because of the risk of inducing generalized systemic reactions when high doses are administered.

It would therefore be highly desirable to be able to administer high doses of antigen which would raise adequate levels of blocking antibody with concomitant symptomatic relief, without risking induction of systemic allergic reactions during treatment. Attempts to achieve this goal have included the use of antigen-oil emulsions,¹⁴ alum-precipitated antigens,^{15, 16} and physical and chemical treatment of antigens.¹⁷⁻²⁰

A seemingly rational approach to improving immunotherapy would be to use an antigen which has been modified to reduce or abolish its native allergenic reactivity while largely retaining its native capacity to produce blocking antibodies able to cross-react with and neutralize the native allergen. Such allergen derivatives have been produced by Marsh and associates,^{21, 22} who converted rye Group I, the major allergenic component of rye grass pollen, into "allergoids" by using mild formalin treatment. The potential of allergoids in the immunotherapy of allergic (atopic) diseases is theoretically based on the premise that they could be administered in high dosage with negligible risk of systemic reactions and with concomitant immunologic protection from allergic symptoms.

The purpose of the present study is to extend the allergoid concept to a mixed grass pollen extract of a type commonly employed in conventional immunotherapy.

*No controlled experiment quantitatively correlating symptomatic relief with antigen dosage has yet been performed. However, there is a very clear difference between symptom scores at the 10,000 PNU and 1,000 PNU levels of ragweed; treatment with the 1,000 PNU level shows no significant difference from treatment with placebo.

MATERIALS AND METHODS

Dialyzed pollen extracts

A five grass pollen mix, containing equal weights of Italian rye (*Lolium multiflorum*), meadow fescue (*Festuca elatior*), orchard (*Dactylis glomerata*), timothy (*Phleum pratense*) and June (*Poa pratensis*) grass pollens, was kindly supplied by Hollister-Stier Laboratories. The pollen (20 Gm.) was defatted with peroxide-free ether and extracted with 0.125M NH_4HCO_3 for 18 hours at 4°, in a manner similar to the previously described for rye pollen.²³ The extract was centrifuged and a portion of the supernatant (100 ml.) dialyzed extensively against 0.002M NH_4HCO_3 (4 × 18 L.) and finally against distilled water (18 L.) for a total of 4 days. The dialyzed extract was clarified by ultracentrifugation and lyophilized, yielding 790 mg. solids (water content about 2 per cent). This extract was used for subsequent formalinization treatment and all biological assays. All concentrations are expressed in terms of milligrams of dialyzed, lyophilized solids per milliliter. One milligram of this extract was found to be equivalent to 4,300 PNU, protein nitrogen being measured with the use of the Nessler procedure on material which was precipitable from a solution containing 5 per cent (W/V) trichloroacetic acid.

Dialyzed extracts of the individual pollens of the grass pollen mix were prepared in a similar manner.

Rabbit antisera

Antisera were prepared by hyperimmunizing rabbits with individual dialyzed crude pollen extracts, with the formalinized product (MGF), prepared using 0.36M HCHO, or with purified rye grass antigens of Group I or Group II.^{23, 24} All antigens were injected intramuscularly as emulsions in complete Freund's adjuvant. Seven to nine injections were administered every 2 weeks using 10 mg. of crude extracts or 2 mg. of the purified allergens. Sera taken 10 days after the final injection were used for immunodiffusion analyses of modified and native mixed grass extracts, according to the micro-Ouchterlony method. Anti-Group I serum was also used to determine the Group I content of the mixed grass extract by the radial immunodiffusion method of Mancini and associates.²⁵

Formaldehyde modification

The methodology used for formalin treatment (formalinization) of dialyzed mixed grass pollen extract was basically the same as previously described²¹ for the treatment of purified rye Group I allergen, except for changes in allergen concentration and some formaldehyde concentrations. The dialyzed extract (2.00 mg. per milliliter) was treated under sterile conditions with various concentrations of formaldehyde, in the presence or absence of low molecular weight reactive additives,* in 0.1M phosphate buffer at pH 7.50 ± 0.05 (initial pH) containing Merthiolate (1:10,000). The compositions of the reaction solutions are summarized in Table I. All solutions were incubated for a total of 32 days at $32^\circ \pm 1^\circ$, samples being removed from these solutions at 1, 8, 16, and 32 days in order to record pH changes. Following the 32 days' incubation, all samples were dialyzed against 0.1M phosphate buffer at pH 7.50 in order to remove unreacted formaldehyde and additives; the resultant solutions were stored frozen at -20° .

Skin testing

Direct skin testing of grass pollen-sensitive subjects was the main method used to compare the allergenicities of the formalinized preparations relative to the native extract.

Scratch method. This method was used to compare the skin reactive properties of a large number of different formalinized allergen preparations relative to the native mixed grass extract from which they were derived. Twenty-one known grass-sensitive patients of pediatric age group were tested on the back, with the use of a Center Scarifier (Center Labs. Inc., Port

*Such additives became incorporated chemically into the resultant formalinized derivatives.²¹

TABLE I. Compositions of reaction solutions used in the formalinization process

Preparation	Dialyzed mixed grass (mg. dry solids/ml.)	Formaldehyde concentrations (M)				Additive (0.05 M)
		a	b	c	d	
MGC*	2.00	None added				None
MGF	2.00	0.06	0.12	0.24	0.36	None
MGF-adipamide	2.00	0.06	0.12	0.24	0.36	Adipamide
MGF-lysine	2.00	0.06	0.12	0.24	0.36	L-Lysine
MGF-ornithine	2.00	0.06	—	—	—	L-Ornithine
MGF-arginine	2.00	0.06	—	—	—	L-Arginine
MGF-diaminobutane	2.00	0.06	—	—	—	1,4-Diaminobutane

All reaction solutions were buffered with 0.1M NaH_2HPO_4 - NaH_2PO_4 buffer + Merthiolate (1:10,000), adjusted to pH 7.50 ± 0.05 , and sterilized by membrane filtration prior to incubation at $32^\circ \pm 1^\circ$ for 32 days. Prior to incubation, all solutions contained 8,600 PNU per milliliter of material precipitable from 5 per cent trichloroacetic acid, and 155 μg Group I allergen per milliliter (determination using anti-rye Group I serum²⁵).

*MGC (first mixed grass preparation, control) will be used to designate a sample incubated without formaldehyde and MGC₀ will denote the corresponding native extract which has not been incubated.

Washington, N. Y.). Antigen solutions (1 mg. per milliliter in 50 per cent glycerine-phosphate-saline buffer at pH 7.5, containing Merthiolate 1:10,000) were applied to the uniform circular scratches. Reactions were read at 20 minutes. Skin reactions were graded as negative or 1+ to 5+, according to the size of the wheal produced and the presence of pseudopodia: 0 = reaction given by negative control (50 per cent glycerine in buffered saline solution) with no wheal detectable; 1+ = average wheal diameter 3 to 5 mm. with no pseudopods; 2+ = average wheal diameter 6 to 8 mm. with no pseudopods; 3+ = average wheal diameter 9 to 11 mm. with at least one pseudopod; 4+ = average wheal diameter 12 to 15 mm. with more than one pseudopod; 5+ = average wheal diameter 16 mm. and over with multiple pseudopods.

Intradermal method. The skin-reactive properties of three formalinized preparations, which were selected for their low reactivity by scratch testing, were more quantitatively assayed relative to native mixed grass extract by intradermal testing in 11 of the previously tested patients. The native and the formalinized extracts were prepared in buffered saline containing 0.05 per cent (w/v) human serum albumin (HSA) as a stabilizer. The solutions were sterilized by cellulose membrane filtration and appropriate dilutions were prepared in the above diluent. Reactions were measured 15 minutes after injection of 0.02 ml. of the solutions and were graded by the same criteria given above. A 3+ skin reaction being selected as the basis for comparison of allergenic reactivity, the allergenicity of a formalinized preparation relative to the native extract was expressed as the ratio of allergoid and allergen concentrations giving this reaction.

Bronchial inhalation challenge experiments

A second comparative assay of the allergenicity of formalinized relative to native extracts was performed by direct bronchial challenge of sensitive subjects with the antigen preparations.

Five children (age 9 to 16 years) from the group of eleven who had been skin tested by the intradermal method volunteered for bronchial inhalation challenge experiments. These children had shown marked skin test reactions to the native grass pollen extract by both the scratch and the intradermal techniques, with significant reduction in their skin reactivity to the allergoid preparation. They had received no bronchodilators, antihistamines, or steroids in any form for at least 48 hours prior to the challenge. All were free from respiratory symptoms and had no auscultory findings of asthma at the outset of the bronchial challenge experiments.

One of the least allergenic formalinized preparations (MGF-lysine, prepared with 0.36M HCHO) and the native allergen (MGC₀) were selected for comparison by the bronchial

challenge method. Appropriate sterile dilutions of the antigens were prepared in the saline-HSA diluent used for intradermal skin testing. The antigen solutions in 1 ml. volume were aerosolized using a DeVilbiss nebulizer propelled by compressed air and adjusted to a flow rate of 15 L. per minute. The initial concentration of antigen or formalinized preparation used in inhalation was determined by that concentration which gave the slightest (1+) positive skin reaction in skin testing. Twofold concentrations were used thereafter (Table II). The experiments were performed in the presence of a physician, who was aware of the nature of the preparations being inhaled, and a pulmonary physiologist, who, like the patient, was not aware which antigen preparation was being tested. There were frequent control aerosolizations using sterile saline diluent at the onset of the challenge and during the course of the sessions. Spirometric determinations were done 5, 10, and 20 minutes after aerosolization of each concentration of antigen. Any subjective symptoms and/or auscultory signs of asthma (wheezing) were an indication for treatment with bronchodilator and interruption of the testing, which was resumed under base-line conditions on a subsequent session (minimum interval of 24 hours). The pulmonary function determinant used to quantitate an equivalent bronchial reaction to formalinized and native allergen solutions was maximum mid-expiratory flow rate, a sustained reduction of 20 per cent below the base-line value for each patient being the parameter of choice.

Each patient was tested first with the formalinized and then with the native allergen. The allergenicity of formalinized relative to native allergen was determined from the ratio of the antigen concentrations causing the same 20 per cent reduction in maximum mid-expiratory flow rate.

Immunization of guinea pigs

Four groups of 6 guinea pigs of the Campbell-Trapani strain were closely matched according to weight and sex. The guinea pigs were maintained under as near identical conditions as possible throughout the experiment. The four groups were immunized respectively with the native antigen MGC₀ and antigens MGF, MGF-adipamide, and MGF-lysine, all of which had been formalinized with 0.36M formaldehyde (Table I). Each animal received as a first course 500 μ g of antigen, emulsified in Freund's complete adjuvant, administered in the hind footpads. The second course, given 34 days later, was with 100 μ g of emulsified antigen given intramuscularly. Following a month's rest, the animals received four further courses of 40 μ g of antigen in saline, administered intradermally at weekly intervals. All animals were bled 10 days after the final injection. Equal volumes of sera from each animal within a group were pooled.

Passive cutaneous anaphylaxis (PCA) tests

Twelve nonimmunized guinea pigs received 13 intradermal injections (0.1 ml.) in their backs comprised of: (1) all 12 animals received 6 serial dilutions of anti-MGC₀ (anti-native antigen serum); (2) the 12 animals were divided into 3 groups of 4, each of which received 6 serial dilutions of one of the three anti-formalinized antigen serum pools; and (3) all animals received undiluted serum from a nonimmunized guinea pig as a control. Serum dilutions were in the range 1:2 to 1:600, appropriate dilutions for each part of the experiment having been determined in preliminary tests on other guinea pigs. Four hours later, 2 guinea pigs from each group of 4 were challenged by intracardiac puncture with native antigen (500 μ g in 1 ml. of 0.5 per cent Evans blue dye); the remaining pairs were similarly challenged with the formalinized antigen (500 μ g in Evans blue) homologous with the antiserum injected previously. The animals were put to death and skinned 45 minutes later and the reactions graded on a scale of 1 to 10 according to the areas and intensities of bluing on the underside of the skin. In this manner, we could observe cross-reactions between native antigen (MGC₀) and each of the antisera to the formalinized derivatives and cross-reactions between each of the formalinized antigens and antiserum to native antigen. Estimates of cross-immunogenicity could then be made from the ratio of the dilutions of heterologous and homologous antisera which gave equivalent bluing reactions of intermediate intensity in the skin of the same guinea pig.

TABLE II. Results of skin tests by the scratch method using native and formalinized mixed grass glycerine-saline

Patient	Commercial MG*	Controls					
		MGC ₀	MGC	MGF			
		0	0	0.06†	0.12	0.24	0.36
G. Y.	3	4	3	0	1	0	0
R. N.	3	1	0	0	0	0	1
G. S.	3	3	1	0	Not done		
H. K.	4	4	2	0	Not done		
F. R.	4	4	4	0	0	0	0
S. E.	4	3	3	0	0	1	0
H. Z.	5	4	3	1	0	0	0
L. Y.	3	3	3	0	0	0	0
M. S.	4	3	1	0	0	0	0
W. L.	4	4	5	0	0	0	0
L. N.	4	4	4	0	0	0	0
K. G.	5	5	3	1	2	0	0
H. E.	5	3	3	2	0	0	1
T. O.	5	4	3	3	1-2	1-2	0
C. E.	4	3	3	2	1	0	1
B. L.	4	3	3	1-2	1	1	1
M. O.	5	4	4	3	3	2	0
W. N.	4	3	3	3	2	1	1
S. N.	4	4	4	3	3	3	3
C. D.	5	4	4	4	3	4	3
P. S.	5	5	5	4	2	3	3

See text for description of skin test grading and Table I for nomenclature. Only reactions greater 0 to 1+.

*The commercial mixed grass (Hollister-Stier Labs.), 5 per cent w/v extract, was equivalent to †Formaldehyde concentration (M).

RESULTS

Following 32 days' incubation at 32°, the formalinized grass pollen derivatives were obtained as clear yellow solutions. In many instances, particularly those in which basic additives were present in the incubation mixtures, small reductions in pH of between 0.1 and 0.4 unit were observed. Such pH changes are attributed to the loss of basic groups, occurring as a result of reaction of formaldehyde with basic side chains of pollen components and additives.²¹

Comparative allergenicity by skin testing and bronchial challenge

The results of skin tests by the scratch method are shown in Table II. The patients have been arranged approximately in order of the number and intensity of their reactions to the formalinized extracts. The patients usually had marked skin reactions to both of the positive control preparations (the non-incubated sample, MGC₀, and the incubated sample, MGC) and a commercial mixed grass preparation of higher concentration. Incubation of the pollen extract without formaldehyde did not appear to cause any substantial reduction in skin reactivity. Three of the formalinized derivatives, MGF-ornithine, MGF-arginine, and MGF-diaminobutane, all prepared using 0.06M HCHO, were

allergen solutions containing 1 mg. of nondialyzable pollen solids per milliliter in 50 per cent

Formalinized allergen preparations										
MGF-adipamide				MGF-lysine				MGF-ornithine	MGF-arginine	MGF-diamino-butane
0.06	0.12	0.24	0.36	0.06	0.12	0.24	0.36	0.06	0.06	0.06
0	0	1	0	0	0	0	0	0	0	0
0	1	0	0	0	0	0	0	1	0	3
0	Not done			0	Not done			Not done	0	1
0	Not done			0	Not done			Not done	0	3
0	0	0	0	1	0	0	0	2-3	2	3
1	1	0	0	1	0	0	0	0	3	2-3
0	0	0	0	2	1	0	0	2	3	4
0	0	0	0	2-3	1	0	0	0	3	2
0	1	1	0	2	1	0	1	1	1	1
0	0	1	1	4	0	0	0	0	3	0
0	0	0	1	3	2	0	0	3	3	4
3	2	1	0	3	0	0	0	0	4	4
2	2	0	0	3	1	1	0	2	3	4
1-2	1-2	0	0	1-2	0	0	0	1	3	4
1	0	0	0	3	1-2	0	0	0	0	3
1	0	0	0	3	3	0	0	0	3	4
3	3	0	2	4	3	0	0	3	5	5
3	2-3	0	1	2	3	1	0	2-3	3	4
2-3	3	3	2	3	3	3	0	0	4	4
4	4	3	3	5	4	4	3	4	5	4
3	3	3	3	3-4	3	2-3	1	3	5	5

than 1 plus are considered to be significant. Control reactions using glycerine-saline were 0 or approximately 4 mg. of nondialyzable pollen solids per milliliter.

highly allergenic in many people. The three other types of formalinized preparations, MGF, MGF-adipamide, and MGF-lysine (particularly MGF-lysine), were also rather allergenic in a substantial number of patients when 0.06M formaldehyde had been used for formalinization. However, with increasing concentrations of formaldehyde used in chemical modification, the incidence of significant skin reactions (greater than 1+) was reduced roughly in proportion to the formaldehyde concentration. The three derivatives prepared with 0.36M formaldehyde usually showed insignificant allergenic activity by scratch tests at concentrations of 1 mg. per milliliter. However, there were a few exceptions in some highly sensitive individuals, shown at the bottom of Table II, for whom the antigen concentrations were too high to discriminate between the skin reactivities of the various preparations.* These three formalinized preparations were subsequently examined by the more quantitative intradermal method, and one of them (MGF-lysine) was used for bronchial challenge experiments (Table III).

Depending on the tested subject, the allergenicity ratio (formalinized/native) by intradermal skin test varied between 200 and 2,000 with the ex-

*See scratch and intradermal tests for Subjects C. D. and P. S. (Tables II and III).

TABLE III. Comparative allergenicity of formalinized preparations relative to native mixed grass extract (MGC₀) by intradermal skin testing and bronchial challenge assay

Patient	Intradermal skin test			Bronchial challenge		
	Antigen concentration giving 3+ reaction (PNU/ml.)		Allergenicity ratio, formalinized/native	Antigen concentration giving 20% reduction in MMEFR (PNU/ml.)		Allergenicity ratio, formalinized/native
	Native	Formalinized*		Native	Formalinized*	
L. N.	1.0	1,000	1,000	300	10,000	30
J. S.	0.1	50	500	50	5,000	100
M. O.	1.0	1,000	1,000	25	7,500	300
T. O.	1.0	1,000	1,000	50	10,000	200
F. D.	5.0	1,000	200	75	10,000	130
B. L.	5.0	1,000	200			
H. E.	5.0	1,000	200			
H. Z.	1.0	1,000	1,000			
S. E.	0.1	100	1,000			Not determined
C. D.	0.01	10	1,000			
P. S.	0.01	20	2,000			

MMEFR = Maximum mid-expiratory flow rate.

*Three formalinized preparations, MGF, MGF-adipamide, and MGF-lysine, all prepared using 0.36M formaldehyde, gave indistinguishable reactions by skin test. Only one of these preparations (MGF-lysine) was later used for bronchial challenge experiments.

tremely sensitive subjects, C. D. and P. S., having ratios of 2,000 and 1,000, respectively.

Using maximum mid-expiratory flow rate as the parameter to assess bronchoconstriction, we observed that between 30 and 300 times as much MGF-lysine as native allergen was required to induce the same sustained reduction of 20 per cent in the flow rate. While the formalinized/native allergenicity ratio varied from patient to patient by the bronchoprovocation test, it was found to be consistently lower than that observed by skin testing in the same individual.

Immunodiffusion assays

By qualitative immunodiffusion analysis, the formalinized pollen extracts were found to cross-react partially with rabbit antisera against various crude and purified pollen antigens. In general, the greater the residual allergenicity of a formalinized derivative, the greater was the degree of cross-reaction observed. The ability of different antigens in the native crude grass extract to precipitate with the antisera was lost to different extents as a result of formalin treatment. In the derivatives of very low residual allergenicity (MGF, MGF-adipamide, and MGF-lysine, prepared using 0.36M HCHO), certain antigen's ability to precipitate with anti-native crude grass sera was destroyed (within the limits of detectability); other antigens were only partially altered (Fig. 1). When the same formalinized preparations were examined against antisera to the potent rye grass pollen allergens of Groups I and II, partial loss of precipitation was observed for Group I and apparently complete loss for Group II (Fig. 2). An antiserum raised against the formalinized preparation MGF was found to give at least two distinct precipitin lines against all the most extensively formalinized preparations, the strongest line of which was shared with the native extract, MGC₀ (Fig. 3).

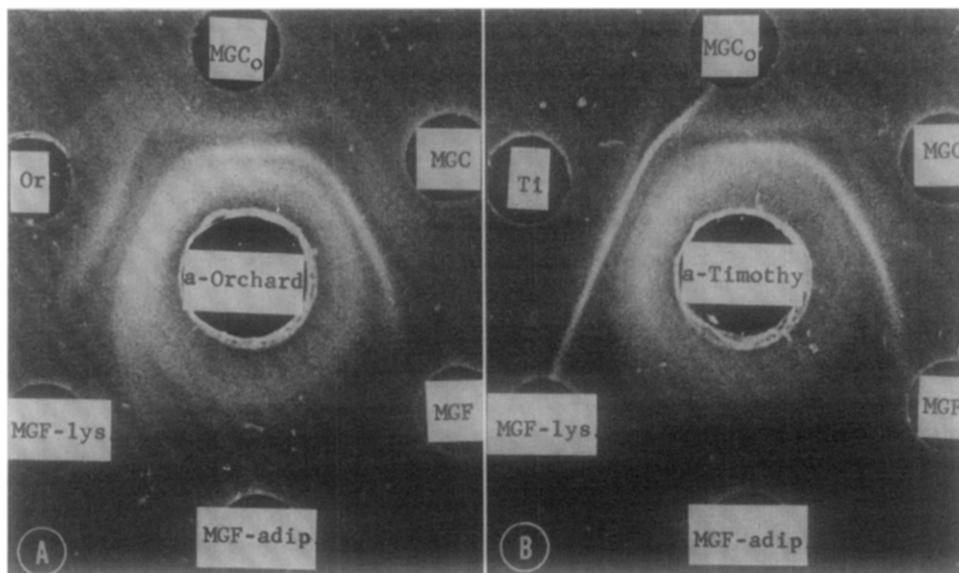


FIG. 1. Immunodiffusion experiments showing partial cross-reaction between native and formalized grass pollen antigens ($15\mu\text{l}$ at 2 mg. per milliliter). All formalized antigens were prepared using 0.36M HCHO (see Table I for nomenclature). The antisera were hyperimmune rabbit anti-native dialyzed orchard pollen extract and anti-native dialyzed timothy pollen extract. Similar results were obtained using anti-rye, anti-June, and anti-fescue sera.

PCA tests

The results of the PCA experiments are summarized in Table IV. Two of the most extensively formalized preparations induced guinea pigs to produce antibody which was able to cross-react with native antigen (MGC_0), as indicated by positive PCA reactions. The ability of the formalized preparations to induce such cross-reactive antibody was in the order: $\text{MGF} \approx \text{MGF-adipamide} \gg \text{MGF-lysine}$. Conversely, the native antigen induced very little, if any, antibody which cross-reacted with the three formalized preparations, although the reciprocal serum titers giving minimal PCA for the homologous reaction, MGC_0 versus anti- MGC_0 , were high (160 to 600).

DISCUSSION

We have shown that it is possible to modify a crude mixed grass pollen extract, of a composition frequently used in the immunotherapy of grass pollinosis, such that its residual allergenicity is reduced very substantially (200- to 2,000-fold by skin test) without extensive loss of its original immunizing properties, as determined by immunization of guinea pigs. Such derivatives, termed "allergoids," have previously been prepared from the highly purified major rye grass allergen, Group I.²¹

In the present study, using crude dialyzed mixed grass extract at 2 mg. per milliliter, the three least allergenic derivatives were prepared with the use of

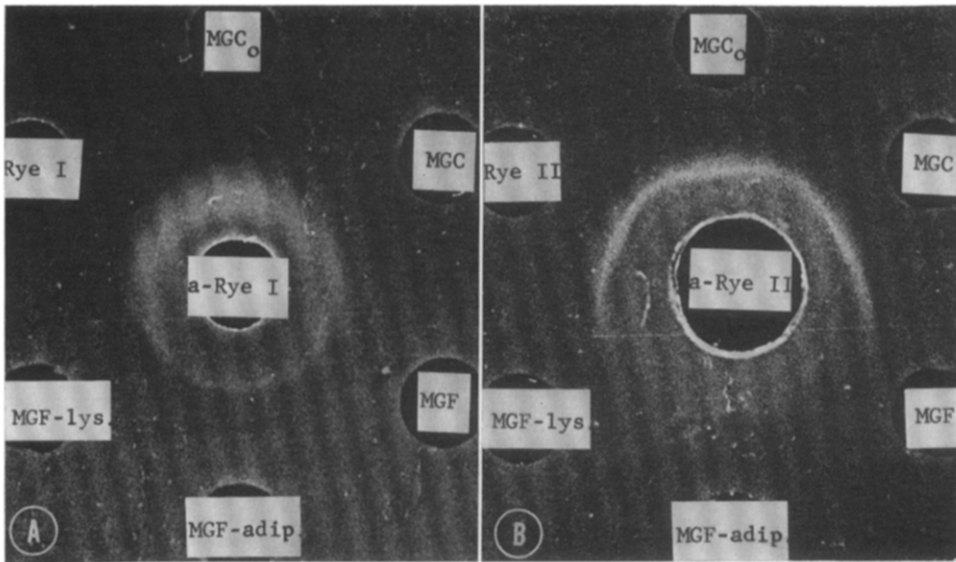


Fig. 2. Immunodiffusion analysis of native and formalized grass pollen antigens against: **A**, rabbit anti-native rye Group I and **B**, rabbit anti-native rye Group II. All formalized antigens were prepared using 0.36M HCHO.

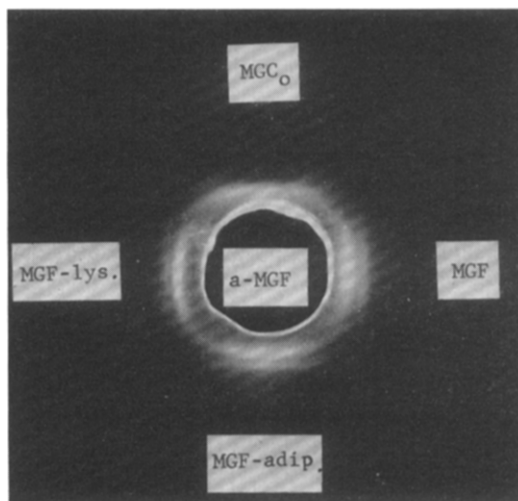


FIG. 3. Immunodiffusion analysis of native and formalized grass pollen antigens against rabbit anti-MFG (mixed grass formalized using 0.36M HCHO). All formalized antigens were prepared using 0.36M HCHO.

0.36M formaldehyde, which is a threefold greater ratio of formaldehyde to allergen on a dry weight basis (or a ninefold higher ratio on a protein nitrogen basis) than previously used to prepare Group I allergoids; other conditions of chemical modification were similar for the crude and purified grass allergens. The three least allergenic mixed grass derivatives, MGF, MGF-adipamide, and MGF-lysine (respectively prepared using formaldehyde alone, formaldehyde plus the additive adipamide, and formaldehyde plus lysine—Table I)

TABLE IV. Cross-immunogenicity between native and formalinized mixed grass preparations determined by PCA tests of guinea pig antisera in normal guinea pigs

Guinea pig No.	Challenging antigen (500 µg)	Heterologous antiserum	Reciprocal antiserum dilution giving an equivalent PCA reaction*		Ratio reciprocal dilutions at equivalent reaction, heterologous antiserum / homologous antiserum × 100 %
			Anti-homologous antigen	Anti-heterologous antigen	
1;2	MGC _o	a-MGF	160;240	40;40	25;17
3;4	MGC _o	a-MGF-adipamide	160;320	20;80	13;25
5;6	MGC _o	a-MGF-lysine	600;160	<2;<2	<0.3;<1
7;8	MGF	a-MGC _o	80;160	<2;<2	<2;<1
9;10	MGF-adipamide	a-MGC _o	80;320	<2;<2	<2;<0.6
11;12	MGF-lysine	a-MGC _o	80; 80	<2;<2	<2;<2

The ratios given in the right hand column give a measure of the cross-immunogenicity between native and formalinized antigens. All animals received 8 dilutions each of antisera homologous and heterologous to the challenging antigen. Controls of undiluted normal guinea pig serum were all negative.

*Reactions were graded on a scale of 1 to 10 according to area and intensity of bluing on the underside of the skin. For any given animal, a reaction graded as either 4, 5, or 6 was used to compare equivalent reactions toward homologous and heterologous antisera.

showed similar abilities to cross-react with rabbit anti-native antigen sera in immunodiffusion experiments, but they differed in their capacities to induce guinea pigs to produce antibody cross-reacting with the native antigens. Only preparations MGF and MGF-adipamide induced good PCA titers of such cross-reactive antibody, while MGF-lysine failed to do so.* This result is somewhat surprising since the corresponding "lysine" derivative of Group I allergen, although possessing very low allergenicity, had previously been found to be particularly efficacious in inducing guinea pigs to produce antibody which cross-reacted strongly with native Group I, determined by neutralization of allergen-mediated histamine release from allergic human leukocytes as well as by PCA tests in normal guinea pigs.²¹ Conversely, the Group I derivative prepared with adipamide was a poor producer of antibody which cross-reacted with native Group I.

When nonallergic man was immunized with native Group I antigen or with "normal" or "lysine" allergoids of Group I (i.e., allergoids prepared using formaldehyde alone or formaldehyde plus lysine), it was shown that the allergoids were almost as effective in producing allergen-neutralizing blocking antibody as was the native antigen.²² Thus, the relative immunogenic properties of the Group I antigens were similar in both guinea pigs and man. By analogy

*We have subsequently tested formalinized derivatives of mixed grass incorporating widely different proportions of lysine. Using formaldehyde/lysine molar proportions of less than 0.36: 0.20, the derivatives were found to be highly skin reactive. All mixed grass derivatives prepared using ratios of 0.36:0.05 and 0.36:0.10 were weakly skin reactive but did not induce significant production of antiallergen antibody in guinea pigs. Four subsequent preparations of mixed grass derivatives prepared using 0.36M formaldehyde alone have proved to be similar in all respects to preparation MGF.

with these results with the purified grass antigens, we feel that it is reasonable to designate the crude grass derivatives MGF and MGF-adipamide as allergoids, although further experiments including induction of "blocking antibody" responses in guinea pigs and immunization of allergic human beings are necessary to justify this designation.

The most extensively formalinized derivatives showed at least a 200-fold reduction in allergenicity relative to the native allergen by intradermal skin tests on 11 individuals (scratch tests in a further 10 subjects appear to be consistent with this finding). This result strongly suggests that the most important allergenic determinants in the grass pollen extract are protein in nature, since formaldehyde modification primarily affects protein moieties; this finding further substantiates a similar conclusion reached in other studies of grass allergens.²⁶⁻²⁸

The allergenicity ratios (formalinized/native) for the most highly formalinized derivatives were different for intradermal skin tests and bronchoprovocation experiments performed in the same individuals. These results may reflect inherent differences in the two target organs or technical difficulties in quantitating the clinical responses, particularly the bronchoprovocation test. It is uncertain how much antigen reaches the lungs and to what extent the dose producing bronchoconstriction represents that which is administered immediately prior to constriction or, in part, a cumulative effect of all previous antigen challenges made on the same day. Nonetheless, bronchoprovocation testing clearly demonstrates a significant reduction in the allergenicity of the mixed MGF-lysine preparation. From the results of skin testing it seems likely that the allergoids, MGF and MGF-adipamide, also would have shown reduced activity in bronchial provocation, but this requires confirmation in view of the differing allergenicity ratios obtained by bronchial and skin tests and the lower immunogenic activity of MGF-lysine in guinea pigs.

The PCA experiments showed that the formalinized antigens were much more effective in inducing antibody cross-reacting with native antigen than was the native antigen in inducing antibody cross-reacting with the formalinized antigens. This result may be due to the formalinized derivatives being better immunogens than the native antigen.

In conclusion, the present experiments suggest that mixed grass allergoids of potential use in allergic human beings can be prepared by appropriate formalin treatment of dialyzed mixed grass extracts. Further development and testing of such derivatives are currently in progress.

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