
Standardized Extracts Modified Allergens—Allergoids

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Historical Review

Conventional immunotherapy of human allergic disorders as introduced by Freeman and Noon¹ involves injection with gradually increasing doses of extracts of the materials to which the individual is sensitive. Since then, immunotherapy has been widely used and its efficacy and specificity was proved in several controlled clinical studies where appropriate patients and appropriate antigens were used.²⁻⁴ The extent of symptomatic relief was improved by increasing the total dosage of antigen administered and was usually accompanied by an increase in specific IgG-blocking antibody levels in the serum⁵ and changes in other immunologic parameters.⁶ However, the high antigen doses required are often difficult to achieve, particularly in highly sensitive individuals, due to associated general anaphylactic reactions. Thus, one usually accomplishes only partial relief with the risk of general allergic reactions along the way.

Different approaches have been undertaken to improve immunotherapy, either by modifying the antigens or the method of administration to the patient. A seemingly rational approach to improving immunotherapy would be to use an antigen that has been modified in order to reduce or abolish its native allergenic reactivity while largely retaining its native capacity to produce blocking antibodies that are able to cross-react with and neutralize the native allergen.

In the production of vaccines, formaldehyde has long been used to convert bacterial and other proteinaceous toxins into virtually nontoxic toxoid derivatives, capable of immunizing protectively against the corresponding native toxins.^{7,8} In 1935, Carter⁹ claimed to have "detoxified" pollen extracts in a similar manner, but presented no experimental data. Stull et al,¹⁰ probably followed a similar methodology to that of Carter, treated crude aqueous ragweed and timothy grass pollen extracts with dilute formalin, but were unable to produce satisfactory derivatives. They concluded that the best immunogens were "fresh extracts," ie, extracts containing antigens that were as close as

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possible to their native state. More recently, Naterman^{11,12} formalinized tannic acid-precipitated pollen materials, apparently with some success, although the clinical experiments were not controlled and no immunologic analyses were performed.

Marsh and associates^{13,14} successfully applied the concept of modified allergens to immunotherapy. They converted *Lol p I** (rye Group I), the major allergenic component of *Lolium perenne* (rye grass) pollen, into various "allergoid" derivatives by using mild formalin treatment. An allergoid was defined as having a greatly reduced allergenic reactivity compared with the native allergen from which it was derived, while retaining to a high degree other desirable properties characteristic of the native allergen, including the capacity to induce a synthesis of allergen-neutralizing IgG-blocking antibodies in animals and humans and to protect atopic subjects from experiencing allergic symptoms following allergen exposure. The potential of allergoids in the immunotherapy of allergic diseases is therefore based on the theoretical premise that they could be administered in high dosages with negligible risk of systemic reactions and with concomitant immunologic protection against allergic symptoms.

This definition of an allergoid is not limited to formaldehyde-modified products, and extends to other types of chemically and/or physically modified allergens with similar immunologic properties. However, this paper will deal mainly with "formallergoids".¹⁶

Technique of Modification

In early experiments it was shown that both purified *Lol p I* allergen and dialyzed mixed grass pollen extracts could be converted into allergoids by mild treatment with formaldehyde solution.^{13,17} In adapting the original formalinization processes from grass to short ragweed (*Ambrosia artemisiifolia*) pollen allergens, the problem arose that important ragweed allergens, including *Amb a I* (AgE), are markedly heat labile. Thus, in preliminary experiments, it was found that modification of purified *Amb a I* with 0.06-M formaldehyde at 32°C, by procedures analogous to those used for *Lol p I*, resulted in the loss of immunizing properties to a degree considered inadequate for use in the immunotherapy of allergic humans. Clinical studies with dialyzed crude ragweed extract showed an almost complete loss of desirable immunizing properties when the native allergen was treated with either 0.36-M formaldehyde alone or formaldehyde plus L-lysine, as used in modifying grass allergens.¹⁷

For these reasons it was necessary to devise a procedure whereby *Amb a I* (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 more widely applicable and produce more efficacious products. These considerations led to the concept of a 2-step incubation procedure using for-

*The nomenclature recommended by the WHO-IUIS Allergen Nomenclature Sub-Committee¹⁵ will be used throughout this chapter. This nomenclature uses the first 3 letters of the genus and the first letter of the species (italicized), followed by a Roman numeral designating the allergen.

Step 1:	Dialyzed defatted allergen extract 10 mg/ml	2M HCHO, pH 7.5 16 days, 10°C	→ Stabilized intermediate
	– Dilute 4x with phosphate buffer at pH 7.5 –		
Step 2:	Stabilized intermediate 2.5 mg/ml	0.5M HCHO, pH 7.5 16 days, 32°C	→ Allergoid

Figure 1. Production of formallergoids by the 2-step process.¹⁶ Residual formaldehyde is removed by dialysis using Visking or Amicon membranes. The resultant allergoids are stabilized by addition of 50% glycerin or are lyophilized from water solution. (From reference 22.)

maldehyde (Fig. 1).¹⁶ The first step, modification at low temperature, results in a modest degree of intermolecular and intramolecular cross-linking, with the expected small reduction in allergenic activity. Most importantly, this leads to the retention of desirable immunizing potency by stabilizing the native molecular conformation of heat-labile antigens under conditions of minimal thermal denaturation. The cross-linked product from the first step is stable toward thermal degradation at the second step, and further reduction of allergenic reactivity can be achieved with minimal loss of immunizing potency.

Clinical Trials

Allergoids produced by the 2-step process first became available for clinical use in the United States in 1972. The results of a long-term study in ragweed-allergic humans between 1973 and 1978, which compared short ragweed formallergoid with short ragweed allergen,¹⁸ demonstrated a significant clinical superiority of allergoid over allergen following prolonged therapy (ie, by the third year), when all the patients in both groups had attained well-tolerated high individual treatment dosages—200–250 µg *Amb a I* equivalents* (34,000–43,000 PNU) of allergoid or 5 µg *Amb a I* equivalents (1800 PNU) of allergen—administered every 2 weeks. It was possible to administer much higher allergoid doses than allergen doses because of the reduced allergenicity of the allergoid.

These encouraging results led to several further studies, the results of 3 of which will be discussed in some detail. In 2 studies short ragweed immunotherapy was performed in 1978 and 1979¹⁹ and in 1980²⁰ during the 12–20 weeks before the respective ragweed seasons. In the 1978–1979 study, the dosages of both the ragweed allergen and allergoid were pushed to tolerated levels, which varied significantly from patient to patient (Table 1). While the resultant IgG-antibody levels to *Amb a I* in allergoid-treated patients were significantly higher than in allergen-treated patients, the median symptom-medication scores for the 2 groups during the ragweed seasons were not significantly different in either year. The 1978 scores for the allergoid group were, however, significantly lower ($p < .01$) than the scores in a matched

*Ragweed allergoid concentration may be expressed in *Amb a I* (AgE) equivalents based on the *Amb a I* content of the allergen from which the allergoid is derived.

Table 1. Data for 1978-1979 and 1980 Studies of Ragweed Allergen and Allergoid

Year	Treatment	No. of patients	Mean no. of visits/ injections	Geometric mean/range			Posttreatment symptom-medication score Median/range (Arb. units)
				Top dose (μg Amb a I equiv.)	Cumulative dose (μg Amb a I equiv.)	IgG to Amb a I (ng/mL)	
1978	Allergen	19	15/16	1.3/0.1-5.0	5.2/0.5-24	1547/424-8267	4.8/0.6-13.2
	Allergoid	16	6/12	37/1.0-250	177/4.0-983	2952/567-9880	5.1/0.0-10.8
	Placebo ^a	16	5	0	0		8.1/4.6-16.0
1979 ^b	Allergen	17	5/8	2.3/0.3-5.0	4.1/0.3-10.8	1429/439-3736	4.9/0.2-14.5
	Allergoid	15	5/9	83/2.8-450	369/6.7-790	3010/588-9932	3.2/0.9-7.8
1980 ^c	Allergoid (low)	12	6/8	20/all the same	32.6/all the same	1509/492-4714	6.1/0.6-11.1
	Allergoid (high)	7	6/12	123/75-150	221/155-255	3320/1773-12,591	2.4/0.4-5.8

From reference 22.

^aColored solution containing histamine administered in same schedule as allergoid.^bContinuation of the same patients studied in 1978.^cSeven weakly sensitive patients were excluded from the totals.

placebo group tested in a double-blind manner in 1978. Interestingly, the data for the subsets of treated individuals who reached the higher antigen dosage levels in 1978 and 1979, showed a trend toward greater clinical efficacy in allergoid-treated than in allergen-treated patients; in this regard, the results were similar to those found in the 1973–1978 study.

We compared the 1978–1979 results with the 1980 data from further highly sensitive patients treated with high- and low-dose short ragweed allergoid.^{20,21} The 12 individuals, who received low-dose ragweed allergoid (20 µg *Amb a I* equivalents; 5000 PNU top dose), had similar symptom-medication scores during the 1980 ragweed season to those patients treated with allergen or allergoid in 1978. However, the 7 patients treated with high-dose allergoid (123 µg *Amb a I*, 35,000 PNU top dose) had significantly lower symptom-medication scores. The latter scores were similar to those found by year 3 of allergoid treatment in the 1973–1978 study. In comparing the low-dose allergoid treatment group of 1980 with the optimally tolerated allergen treatment group in 1978, systemic reactions were absent in the low-dose allergoid group, but 42% of the allergen-treated patients had systemic reactions. The symptom-medication scores and geometric mean IgG-antibody levels to *Amb a I* were similar in the 2 groups. While the clinical scores were for different years and are not strictly comparable, the data do suggest that low-dose allergoid treatment does provide a kind of therapy whose efficacy is similar to optimally tolerated allergen treatment, without the high risk of systemic reactions. On the other hand, in 1980 high-dose allergoid treatment led to significantly improved clinical efficacy ($p = .04$) and higher antibody titres ($p = .06$), but with a systemic reaction in 1 patient and a higher frequency of delayed local reactions.

In 1980, we also carried out a study of high-dose and low-dose short ragweed allergoid treatment.^{21,22} This was a booster immunization study of 2 groups of 19 patients who had been treated in the 1973–1978 and 1978–1979 studies cited above. Doses were again administered preseasonally, but with only 4 visits to the clinician (Table 2). The high-dose group had significantly lower symptom-medication scores than the low-dose group and higher geometric mean IgG-antibody levels to *Amb a I*. The most interesting comparison is that of IgG-antibody levels for patients who had been treated with optimally tolerated allergen doses in 1978 and 1979, and who were switched either to high-dose or low-dose allergoid in 1980 (Table 3). High-dose allergoid led to a 2.9-fold increase in the geometric mean IgG-antibody level over the 1979 values, whereas the low-dose allergoid resulted in a 2.1-fold increase. There was no significant change between 1978 and 1979, whereas switching from optimally tolerated doses of allergen to either high-dose or low-dose allergoid led to significantly higher IgG-antibody levels toward *Amb a I*.

In conclusion, the data indicate a clear advantage of formallergoid over allergen in the treatment of allergic patients. Low-dose allergoid treatment induced no systemic reactions, but 42% of patients had systemic reactions following an aggressive regimen of allergen therapy; however, the 2 regimens were similarly efficacious. High-dose allergoid treatment was clinically and immunologically more efficacious than low-dose allergoid therapy, but the patients incurred more adverse reactions. As a result of these experiments,

Table 2. Booster Study with High and Low Doses of Allergoid

Allergoid treatment	No. of patients ^a	Mean no. of visits/injections	Geometric mean/range			Posttreatment Symptom-medication score Median/range (Arb. units)
			Top dose (μg <i>Amb a</i> I equiv.)	Cumulative dose (μg <i>Amb a</i> I equiv.)	IgG to <i>Amb a</i> I (ng/mL)	
High-dose	19	4/9	142/40-200	304/115-390	3583/415-32,220	2.2/0.05-8.9
Low-dose	19	4/4	27/10-40	62/30-80	2314/473-6390	5.3/0.011-3

From reference 22.

^aPatients from the 1978-79 study, including patients who had been treated with placebo in 1978 and various allergoids in 1979 (not discussed here), were randomly assigned to the treatment groups.

Table 3. Change in Geometric Mean IgG-Antibody Levels Toward *Amb a 1* Resulting from Switching Patients from Allergen to Allergoid

No. of patients	Geometric mean IgG to <i>Amb a 1</i> (ng/mL)		
	1978 Allergen	1979 Allergen	1980 Allergoid
6	2260	2151	6289 (H) ^a
11	1200	1142	2405 (L) ^b

From reference 22.

Note: For patients previously treated with allergoid in 1978 and 1979, the high-dose allergoid boost in 1980 resulted in a 1.3× increase in IgG and the low-dose boost in a 1.5× decrease in IgG antibody to *Amb a 1*.

^aHigh-dose allergoid in 1980.

^bLow-dose allergoid in 1980.

we are now recommending a more cautious approach toward therapy. Initially, we recommend a low-dose regimen, followed by a gradual progression toward higher and more efficacious allergoid dosages. An important fact that emerged from our 1980 booster study²¹ is that patients undergoing allergen therapy can be switched to formallergoid with significantly better immunologic efficacy and with evidence of better clinical efficacy than allergen treatment.

On the basis of these clinical experiences we recommend that allergoids should be used according to a dosage guide comprising 8–11 patient visits (Table 4). A schedule similar to the first 8 visits of this guide (but extending the number of visits to 10) has recently been successfully used in a double-

Table 4. Dosage Guide for the First Year of Treatment with Allergoid

Visit no.	Protein Nitrogen Units		Allergoid Units	
	Single injection ^a	Subsequent injection	Single injection ^a	Subsequent injection
1 ^b	10	20	0.1	0.2
2 ^c	20	40	0.2	0.4
3	40	80	0.4	0.8
4 ^d	250		2.5	
5	500		5.0	
6	1000		10.0	
7	2000		20.0	
8	4000		40.0	
Protection reached comparable to at least a full course of allergen treatment with aqueous extracts				
9	8000		80.0	
10	16,000		160.0	
11	32,000		320.0 ^e	
Total	63,960		639.6	

^aSpacing between injections: 3–4 weeks between visits 1 and 2, 2–4 weeks between subsequent visits.

^bAt the first visit, if there is no significant reaction to the first injection after 30 minutes, a 2-fold higher dosage will be injected into the opposite arm.

^cAt the second visit, the initial injection will be into either arm. If no significant reaction after 30 minutes, the dose will be increased 2-fold and injected into the other arm.

^dSingle injections will be administered at subsequent visits according to the above schedule, except where large local reactions occur. In such case, the schedule will be modified.

^eMay be administered as 2 separate injections of 160 Units in the case of mixtures of allergoids.

blind therapeutic study of ragweed hay fever by Meriney et al.²³ These workers found that the allergoid was well tolerated and there were no systemic reactions in 10 allergic subjects following allergoid injections. The total symptom medication scores for the allergoid-treated patients during the ragweed season were significantly lower ($p < .01$) than those in 10 matched patients treated with placebo. The allergoid-treated patients also exhibited significant IgG-antibody responses to *Amb a I* ($p < .001$).

After the first year of treatment, patients will require allergoid booster injections only every 3 months,²⁴ administering the highest tolerated dose. This certainly provides an advantage over conventional allergen treatment, both for the patient and the physician. Either one can extend visits to 1 every 3 months where mixtures of allergoids are administered, or one can give more frequent injections of individual allergoids. The former approach is more convenient for the patient, but the latter approach may permit higher, more efficacious dosages of individual allergoids in the patient who is highly sensitive toward multiple allergens.

In our studies, we have shown that formallergoid therapy produces good IgG responses not only toward the major allergens such as ragweed *Amb a I* or grass *Lol p I*, but also toward minor allergens such as ragweed *Amb a III* (Ra3), *Amb a V* (Ra5), and *Amb a VI* (Ra6).²² From a practical standpoint, we have also shown that allergoids can be administered perennially as high dosage mixtures, of the kind likely to be used in practice by allergists. Furthermore, immunizing potency is retained following storage for at least 3 years at 0–5°C.¹⁸ Despite the very high dosages, particularly in patients who started therapy in 1973, we have observed no adverse findings by SMA-12, hematology, or urinalysis^{18,19}; there is also no evidence of circulating immune complexes.²²

First clinical studies with grass pollen allergoids in Europe started in 1978.^{25,26} Since aqueous allergen extracts were no longer being used in most European countries, allergoids had to be compared with alum-adsorbed allergen preparations, which cause fewer side reactions than aqueous allergen extracts. Once again we tried several different approaches to find optimal dosages. In highly grass-allergic patients we used too high of dosages in the beginning, which led to a relatively high frequency of side reactions. However, we were able to solve this problem later on by using lower allergoid dosages. In the second and third year the frequency of systemic reactions diminished considerably.²⁷ Grass pollen allergoid also induced a more rapid increase in IgG antibodies than conventional grass allergens; during the first year of treatment, a plateau level was attained. In the allergen group the same titer was reached only after the third year of treatment.²⁷ Accordingly, allergoids led to a faster relief of clinical symptoms. Also, none of the European studies with grass pollen allergoids showed abnormalities of blood chemistry, liver function tests, blood counts, or urinalysis.

Summarizing the results of all our clinical work, allergoids represent an improved preparation for immunotherapy. Numerous trials have demonstrated that allergoids have a better therapeutic efficacy compared with allergen extracts. About 50 times more allergoid than standard aqueous allergen

can be administered to hay fever patients before symptoms of anaphylaxis ensue.¹⁹ In addition allergoids produce higher allergen-specific IgG titers more quickly than standard allergen.^{19,25,27} The results of one study suggest that T-cell defects could possibly be remitted by treatment with allergoids.²⁸

Recently, as an alternative to aqueous allergoids, alum-adsorbed allergoids have been used in clinical studies.²⁹ In this study it was shown that alum-adsorbed allergoid was tolerated better than alum-adsorbed allergen with respect to systemic reactions. Also, the allergoid group reached a titer of IgG-blocking antibodies after 7 injections, which was reached only after a full course of allergen treatment comprising 11–12 injections. These results provide the prospect of a short-term treatment with alum-adsorbed allergoids.

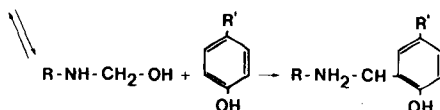
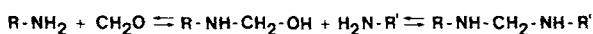
Another alternative mode of application of allergoids with the aim to reduce further the risk of anaphylactic reactions could be the use of the high molecular fraction of allergoids. First promising clinical trials were done by Bousquet et al.³⁰

Characterization of Various Allergoids

Under the reaction conditions described in Figure 1, formaldehyde produces allergoids with low allergenicity and high immunogenicity.^{13,16,17} The formaldehyde reacts primarily with ϵ -amino groups of lysine residues on the surface of the protein molecules, which then interact with tyrosine, arginine, and acid amide residues, leading to both intermolecular and intramolecular cross-linking of the allergen molecules.^{8,13,16,31} One of the possible chemical reactions is shown in Figure 2. These reactions lead to increases in the average molecular weights and to shifts of the mean isoelectric points of the proteins to a more acidic pH. Furthermore, active sites of the allergen could either be modified or concealed within the polymeric structures of the molecules. These accompanying changes in physicochemical properties can be used to characterize allergoids and control allergoid production.

During the development of allergoids we have used several in vitro and in vivo methods to characterize formallergoids; not all of them proved to be applicable as routine assays. Most of our work was done with allergoids of short ragweed pollens, timothy grass pollens, and mixtures of grass pollens. However, the principles of modification have been successfully applied to other allergens, eg, tree pollens (alder, birch, hazel, oak), pollens of *Parietaria judaica* and *Plantago lanceolata*, *Alternaria alternata* (*tenuis*), and the house dust

Figure 2. Possible chemical reactions involved in the modification of allergens by formaldehyde. (From reference 31.)



mite *Dermatophagoides pteronyssinus*. Typical examples for the different methods of characterization will be given.

Physicochemical Properties

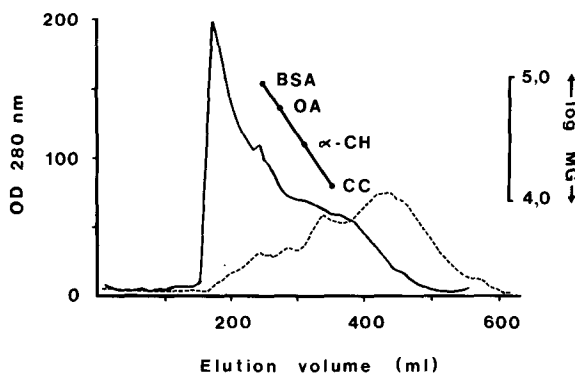
Molecular Weight Distribution

The increased mean molecular weight of formallergoids relative to the allergens from which they were derived can be monitored by size exclusion chromatography, eg, Sephadex G-100 Superfine or high-performance liquid chromatography (HPLC) using TSK columns Bio Sil TSK 250.^{16,32,33} The results of a Sephadex separation of allergen and allergoid prepared from a mixture of 6 different grass pollens are shown in Figure 3. The modification by formaldehyde clearly leads to changes in molecular weight, since the allergoid shows a peak at higher molecular weight than the allergen. Size exclusion chromatography, especially the rapid separation by HPLC, is a fast and reproducible method for in-process control of allergoid production.

Isoelectric Focusing

Due to reactions of the formaldehyde with free amino groups, the net charges of proteins are changed, which is expressed in changes of isoelectric points. An example of comparative isoelectric focusing of timothy allergen and its corresponding allergoid is shown in Figure 4. Most proteins of the allergen have their isoelectric points in the range of pH 3.0 to pH 6.4, with 2 additional bands at pH 7.4 and pH 9.4. The proteins of the allergoid focused within a more confined range of pH 3.0–5.6, and furthermore, fewer bands were found after formaldehyde modification of the allergen. We consider isoelectric focusing as a suitable method to control the modification of the allergen; however, no information is given concerning biologic activity.

Figure 3. Gel chromatography (Sephadex G-100 Superfine) of a grass-pollen allergen (-----) and the corresponding allergoid (—). The column was calibrated using bovine serum albumin (BSA), ovalbumin (OA), α -chymotrypsinogen (α -CH), and cytochrome c (CC). (From reference 32).



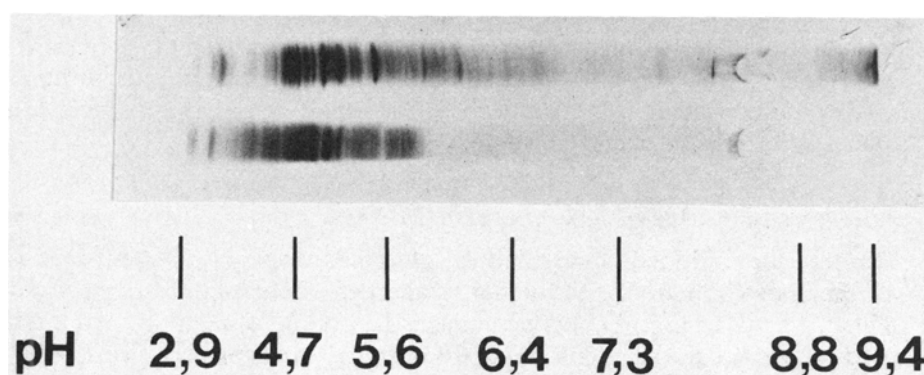


Figure 4. Isoelectric focusing of a grass-pollen allergen and its corresponding allergoid on PAG plate. 60- μ g protein in 20 μ L were focused. (From reference 32).

Changes in Biologic Activity

Enzyme Determination

Extracts from viable pollens contain enzymes that metabolize external and internal substrates essential for pollen tube growth.³⁴ The presence and the concentrations of certain enzymes in allergen extracts have been described as quality criterion for allergen extracts by Bousquet et al.³⁵ It can be assumed that changes in protein configuration due to formaldehyde treatment will also affect the enzymatic activity.

In grass pollen allergens the dominant enzymes were acid phosphatase and leucine-amino peptidase.^{32,33} After treatment of the allergens with formaldehyde most of the enzymes were completely inactivated; only traces of acid phosphatase activity could still be found. There were similar observations in the cases of allergoids derived from *Parietaria judaica*, *Betula verrucosa*, *Dermatophagoides pteronyssinus*, and *Alternaria alternata* (data not published). This lack of enzymatic activity, particularly of proteolytic activity, is largely responsible for the greatly increased stability of allergoid solutions.

Immunogenicity by Radioimmunoassay

One of the claims for allergoids is their retained ability to induce blocking IgG antibodies against the native allergen. To prove this the relative immunogenic potencies of the native and formaldehyde-modified allergens were investigated by their capacity to induce antiallergen antibody production in guinea pigs.^{13,16,17}

Overall, the experiments showed that guinea pigs immunized with formalallergoids produced appreciable amounts of cross-reactive IgG-“blocking” antibody against the native allergen. The mean amounts of antibody produced against the allergoid were usually 30–100% of that produced by the allergen. High levels of antibody production also were observed in cases where rabbits³²

and mice (data not published) were immunized with mixed grass or ragweed pollen allergoids. These findings were later confirmed by studies in humans where various allergoids produced IgG antibodies against *Amb a* I minor allergens of ragweed, and grass pollen allergens.^{18-20,25,27,29}

Allergenicity Assays in Patients (Skin Testing)

Through formaldehyde modification, allergoids display a markedly reduced allergenicity compared with the native allergen. Direct skin testing (intradermal and prick testing) of allergic subjects has been the main *in vivo* method used to compare the allergenicities of the allergoids with the native allergen^{14,17,36} on a quantitative basis. Depending on the tested patients, the allergenicity ratios (allergoid/allergen) by intradermal testing varied between 200 and 2000.¹⁴ These ratios were not so high when different allergoid species were assayed in skin prick testing.³⁶ In taking the mean skin reactions of 12 patients, approximately 90 times more protein of allergoid than of allergen was necessary to elicit comparable wheal sizes.

In vivo tests in patients give valuable hints on the actual degree of modification; however, such tests are time consuming and difficult to perform. Moreover, the results depend on the sensitivity of the tested patient. Also, for ethical reasons, skin tests cannot be used as routine assay for batch release in production. For this purpose they are replaced by suitable *in vitro* methods.

Systemic Anaphylaxis Studies in Rats

Another possibility to compare the allergenicity of allergen and allergoid *in vivo* is their capacity to induce anaphylaxis in sensitized animals.³⁷ In our case sensitized rats were challenged by timothy allergen and timothy allergoid

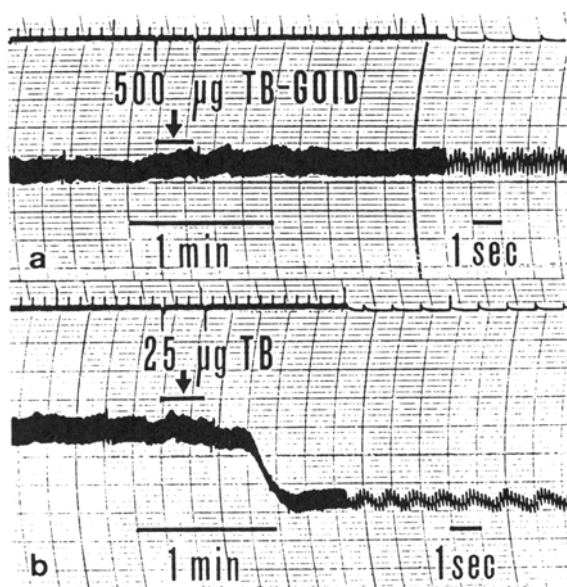


Figure 5. Change in mean arterial blood pressure of a timothy-sensitized rat after intravenous injections of (A) 500- μ g timothy allergoid protein in 0.2 mL phosphate-buffered saline (PBS) and 10 mm later, (B) 25- μ g timothy allergen protein in 0.2 mL PBS. The injections are indicated by arrows. (From reference 37.)

with injections of increasing concentrations in the femoral vein. Changes in the mean arterial blood pressure were registered at the right carotid artery. In our experiments the capacity of allergoids to induce anaphylactic reactions decreased by a factor of approximately 50 when compared with the native allergen (Fig. 5).

Passive Cutaneous Anaphylaxis

As an additional test for measurement of reactivity we used heterologous passive cutaneous anaphylaxis (PCA) titration, again with timothy allergen and allergoid as a model. The capacity of allergen and allergoid to bind mouse IgE antibodies to timothy pollen antigens and to initiate a local anaphylactic reaction was investigated in Sprague-Dawley rats.³⁷ The PCA titer was expressed as the maximum dilution that led to a positive reaction. In our experiments the highest dilution of reaginic mouse serum to timothy pollen that led to a positive PCA reaction was 1:256 and 1:8 for timothy allergen or timothy allergoid, respectively. Thus the allergenicity of timothy allergoid was reduced about 32-fold in this animal model.

Allergenic Activity Measured by Histamine Release from Human Leukocytes

Histamine release from washed human leukocytes of allergic patients is an approved method to assess the allergenicity of allergen preparations.³⁸ Allergenicity ratios for allergen and allergoid from ragweed, timothy, and mixed grass pollens, and *Lol pl* were investigated.^{13,16,17,37,39} Figure 6 shows results of 31 sets of histamine-released assays comparing the allergenic activities of a grass pollen allergoid relative to the native allergen. Approximately a 600-fold mean difference in activity was found between allergen and allergoid,³² however, with large variations (more than 6 log 10) for the individual patients.³⁹ Since the same preparations were used in all cases, the probable reason for the wide variation in allergenicity ratios between individuals is that different individuals recognize different determinants on the various allergen molecules. Presumably these determinants have been altered to various degrees in a given modified allergen preparation. Hence, the degree of reduction in allergenicity can be expected to vary from person to person.

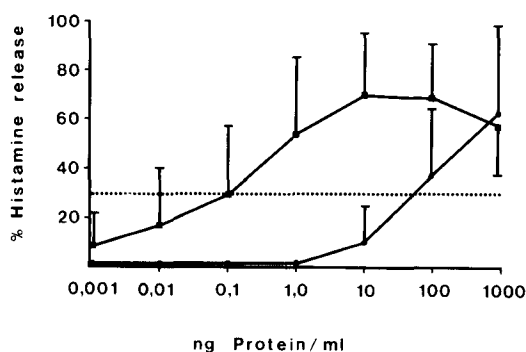


Figure 6. Histamine release from human leukocytes after addition of different amounts of grass pollen allergen (left-hand curve) and its corresponding allergoid (right-hand curve). The vertical bars indicate standard deviation of 31 measurements. (From reference 32.)

Radioallergosorbent (RAST) Inhibition

RAST inhibition is an excellent *in vitro* method to compare the allergenic activities of formaldehyde-modified preparations with the native allergen.³⁹ In a series of experiments this has been done for formalinized *Lol p 1*, ragweed, timothy, and grass pollen preparations.^{13,16,17,32,37,39} An example for allergen and allergoid of a mixture of 6 grass pollens is shown in Figure 7. Approximately 500 times more allergoid protein was needed to achieve the same degree of inhibition as allergen, indicating a 500-fold decrease in allergenicity. The slopes of the inhibition curves for the modified allergen and for the native allergen were not significantly different. Interestingly, a very similar reduction in allergenicity was obtained when allergen and allergoid were compared in RAST-inhibition and histamine-release assays with identical samples using the sera and the leukocytes of the same patients.³⁹ For reasons of practicability and better reproducibility, we consider RAST inhibition as preferential method for routine tests of residual allergenicity.

Further Approaches Toward Standardization of Allergoids

Both allergists and regulatory agencies are demanding standardization of allergen preparations used for the diagnosis and the treatment of allergic patients.⁴⁰⁻⁴³ Because of the ease of routine analysis by RAST inhibition and related technologies, the standardization of allergenic extracts has, so far, been based primarily on assays of relative allergenic reactivity rather than relative immunogenic potency. Some workers have tacitly assumed that allergenic reactivity is directly related to immunogenic potency; therefore, a test such as RAST inhibition was presumed to be suitable for standardization of extracts used in both diagnosis and treatment. In the case of allergoids produced in our laboratories,^{13,14,16,17,32} this assumption has clearly been shown to be invalid. By appropriate chemical modification of allergens, the allergenic reactivity can be greatly reduced, while the immunogenicity is largely retained. Thus, studies of allergoids have brought into sharp focus the inadequacies of current standardization programs, at least for modified allergen preparations. Although the great bulk of allergenic extracts is used for treatment rather than diagnosis, there has been no attempt to provide a standardization of immunogenicity.

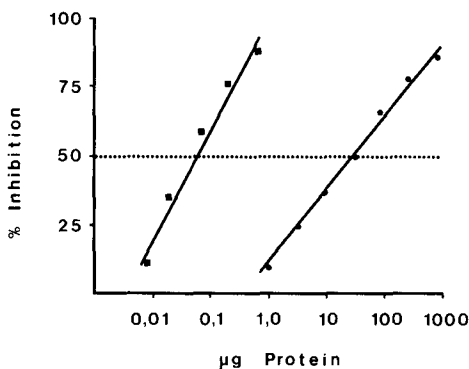


Figure 7. Radioallergosorbent inhibition of a grass pollen allergen (■) and its corresponding (●) allergoid. The results are expressed as percentage inhibition of binding to solid-phase allergens achieved by different amounts of free allergen or allergoid. (From reference 32.)

Standardization Related to Immunogenicity

Since allergoids are not useful in diagnosis, the RAST-inhibition test using an allergen immunosorbent serves primarily as a *safety* test for standardizing batch-to-batch consistency in relation to allergenic reactivity. Quantitative skin testing serves a similar purpose.

Direct measure of immunogenic potency, eg, by immunization of either allergic humans or animals, followed by quantitative assay of IgG-antibody responses, is not practical for routine standardization of allergoid batches. We are, therefore, proposing 2 experimental approaches that depend on utilizing allergoid interaction with appropriate antibodies. The first test has its basis in the proved flocculation methodology used to standardize toxoids.⁴⁴ The second uses RAST inhibition, where the allergoid rather than the allergen is used as the solid-phase immunosorbent.⁴⁵

In the case of the flocculation test, rabbit antiallergoid antibodies are produced by immunizing 3 or more rabbits with an appropriate reference allergoid. Varying dilutions of the reference antiallergoid serum so produced are added to a series of small tubes, and constant amounts of the standard and test allergoids are added. The contents of the tubes are mixed and incubated at 50°C in a water bath. The tubes are examined every 5 minutes by dark-field illumination. The indicator tubes in the case of each allergoid are the ones in which flocculation is first observed. The relative potency of the test allergoid is calculated from the ratio of antiallergoid serum dilutions in the indicator tubes of the reference and test allergoids. We have found that this test correlates well with the relative immunogenic activities of different short-ragweed allergoids, as measured by their abilities to induce anti-*Amb a 1* antibody production in allergic humans (unpublished data).

In the RAST-inhibition procedure, using allergoid on the paper disks, the test is set up in a manner similar to a normal RAST-inhibition experiment, using a serum pool from individuals highly sensitive to the respective allergen. The test allergoid is compared against a reference allergoid in respect to its relative ability to inhibit the binding of the IgE antibody to the allergoid disks. In previous investigations the residual allergenic activity of the reference allergoid has been determined by comparison with the native allergen (Fig. 7). By this approach we avoid the use of high amounts of allergoid protein in RAST-inhibition assays, which may lead to unspecific binding reactions. The sensitivity of the allergoid RAST inhibition is in the same range as the allergen RAST inhibition, ie, approximately 1 µg/mL of allergen and allergoid yields satisfactory inhibition curves.

Concluding Remarks

The nature of formallergoids has been evaluated by several in vivo and in vitro methods. Data from numerous clinical trials point to a clear clinical and immunologic advantage of formallergoid over allergen in the treatment of allergic patients. About 50 times more allergoid than standard aqueous allergen can be administered before symptoms of anaphylaxis ensue, opening the prospect for 2 different approaches to immunotherapy. Low-dose allergoid treatment will give similar efficacy as conventional allergen treatment, how-

ever, without systemic reactions. High-dose allergoid treatment will lead to increased clinical and immunologic efficacy with similar incidences of side reactions than conventional allergen treatment. Booster injections can be given every 3 months, thus reducing the number of injections for a full course of treatment. In the described form allergoids represent an enrichment for allergen immunotherapy.

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