

ORIGINAL ARTICLES

Formalinized pollen protein precipitates with tannic acid or urea in desensitization treatment

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The production and use of three water suspensions of precipitated pollen protein in repository treatment of pollen sensitivity are presented. Two preparations consist of Stull's Fraction I obtained by trichloroacetic acid or by acetylation with acetic anhydride, and converted to insoluble slow-reacting precipitates by tannic acid and formalin. The third preparation is an insoluble pollen protein obtained by urea and formalin. Satisfactory clinical desensitization may be obtained safely with relatively few (two to five) injections of increasing doses (1,000 to 25,000 PNU). The advantages as compared with other forms of repository treatment are discussed. The application of these techniques to other antigens for purposes of immunization is suggested.

The conventional method of pollen desensitization requires about 20 injections of increasing doses of pollen extract. If this amount of antigen, or more, could be injected in a repository form releasing increasing quantities gradually, presumably desensitization could be effected with fewer injections, perhaps even one. Various physical modifications of antigen have been used for this purpose, starting with oil emulsions,¹ mixtures in gelatin,² and alum and tannic acid precipitates.^{3, 4} In recent years, water in mineral oil emulsions⁵ and alum-pre-

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ecipitated preparations⁶ have been studied widely. The purpose of this paper is to present and to stimulate interest in other and new forms of repository therapy.

Aside from the addition of adjuvants such as acid-fast bacilli to enhance immune responses, generally, two methods are used to increase the effectiveness of injected antigens. The repository method slows absorption by changing the *physical* state of the antigen from a solution to an oil emulsion or to an alum precipitate. For immunization when the antigen is relatively nontoxic, this procedure may be sufficient. The second method is to modify bacterial toxins *chemically* by formalin (toxoids) to reduce their toxicity, thus permitting the injection of larger doses. For specific desensitization treatment of clinical allergy, a combination of both physical and chemical modification of allergen would seem more effective than either one alone. Such a combined modification in the form of a precipitate with reduced immediate reactivity was obtained by precipitating pollen protein with tannic acid, and treating the precipitate with formalin.⁷ Variations of this procedure have been tried. The first variation was to use Stull's Fraction I of pollen protein⁸ since it contains the major antigen and is less apt to cause general reactions. Fraction I obtained by precipitation with trichloroacetic acid⁹ has been used to make the formalinized tannate antigen since 1957. In 1959 and 1960, the fraction obtained by acetylation with acetic anhydride and then made into a formalinized tannate was used in the hospital clinic. In 1961 and 1962, a new pollen precipitate with urea and formalin was used for treatment of clinic patients. For representative purposes, the results of grass and ragweed treatment with each of these preparations in 1958, 1960, and 1962 will be presented. Patients with tree sensitivity are excluded because of the small number treated.

METHOD

The pollens used were short ragweed for the August-September season, and equal parts of timothy and orchard grass for the June-July season. After defatting, extractions were made in physiological saline 1 to 20 (w/v) with pH adjusted to 7.2 to 7.4 by 10 per cent NaOH. After 1 to 3 days in the cold, the extracts were paper filtered and used to make the various preparations.

Trichloroacetic acid precipitate of pollen extract as formalinized tannate (TCA Pollen F. T.)

Pollen extract was treated with trichloroacetic acid to 2 per cent concentration, and zinc chloride to 0.1 per cent. After 24 hours in the cold, the precipitate was collected by centrifuging and made to the original volume with saline, with addition of sufficient NaOH to effect solution at about pH 7.0. The solution was made slightly acid with HCl, and a fresh tannic acid solution (10 or 20 per cent) was added to make a concentration of 0.5 or 1.0 per cent, with prompt flocculation of the protein. Formalin to 1 per cent was added. After settling, the supernatant was siphoned off and replaced with 1 per cent formalin in saline. After several such siphonings in the course of 3 to 7 days at room temperature, the nitrogen was determined by nesslerization. Under sterile conditions, the supernatant formalin solution was replaced by saline twice to wash

out the formalin. After washing, at times after light centrifugation, the sediment was resuspended in saline with 0.1 per cent NaHSO_3 to neutralize any remaining formalin, in 0.25 per cent aluminum hydroxide to facilitate flocculation, and in 0.88 per cent phenol as preservative. For clinical use a concentration of 0.25 mg. N per cubic centimeter (25,000 PNU per cubic centimeter) was used.

Acetylated pollen extract precipitate as formalinized tannate (Ac Pollen F. T.)

Pollen extract was treated with acetic anhydride by gradual addition with stirring to a concentration of 2.5 per cent. Zinc chloride to 0.1 per cent was added. The precipitate was collected by centrifugation and processed from then on in the same manner as the trichloroacetic acid precipitate.

Precipitate from pollen extract by urea and formalin (Pollen U. F.)

Pollen extract was treated with urea to 2 per cent and formalin to 5 per cent concentration. Trichloroacetic acid was then added to pH 1, zinc chloride to 0.1 per cent, and polyethylene glycol 400 to 5 per cent was used to obtain a finer precipitate. The mixture was stirred until flocculation appeared. After standing 1 to 3 days at room temperature, the supernatant was siphoned off, the sediment was washed with saline three times and resuspended in saline to the original volume of 1 to 20 extract. The saline medium was previously treated to contain final concentrations of 0.1 per cent NaHSO_3 , 25 per cent glycerin, and 0.88 per cent phenol. A nitrogen determination of the precipitate would include unknown quantities of urea N combined to protein, and, so, would not represent true protein N.

Selection of patients

All patients selected for treatment had histories typical of pollen sensitivity and, definitely, positive skin tests by the scratch method. Patients with mild symptoms responding to medication were excluded.

Administration

Injections were given subcutaneously in the upper arms with the usual hypodermic syringe and needle. The injections were begun about 1 to 3 months before the expected start of the season with the final dose given usually 1 to 4 weeks before the start of the season. Intervals between injections were 1 to 4 weeks or more, usually 3 weeks. Perennial injection intervals were usually 8 to 12 weeks. Patients were tested by the scratch method with pollen dilutions of 1 to 50, 1 to 500, and 1 to 5,000 before treatment to corroborate the diagnosis and to estimate the degree of sensitivity. With the F.T. preparations, the usual dosage schedule was 0.1 ml., 0.35 ml., and 1.0 ml., with the top dose repeated 1 to 3 times. If the skin tests were large or experience indicated a patient was prone to reactions, the starting dose was 0.05 ml. and the top dose was reduced to 0.5 ml. No injections were given during the season. A few patients first reporting during the season were given coseasonal injections, but the results cannot be properly evaluated and these patients are not presented here.

With U.F. 1-20 preparations, the usual schedule was 0.2 ml. and 1.0 ml., with an occasional reduction of this dosage to 0.1 ml. and 0.5 ml. In 1961, most patients received three injections, and in 1962 most patients were given only two injections.

Clinical evaluation

Clinical results were estimated by careful questioning and evaluation of clinical notes. Patients with few or no symptoms and only occasional or no medication were graded as having excellent results. Patients with only an occasional uncomfortable day and slight symptoms readily controlled with medication up to 1 or 2 doses a day, who indicated much improvement as compared with years of no treatment, were considered to have good results. Those with slight improvement were considered to have fair, and those with none, poor results. Patients with excellent and good results were considered to be in satisfactory condition; with fair or poor, unsatisfactory.

In 1962, 30 patients were evaluated carefully and independently by the allergist who treated the patient and by another experienced allergist, with the use of a printed check sheet as a guide. The results were so close that it was decided a single evaluation would suffice.

RESULTS

In 1958, TCA Pollen F.T. preparations were used in the office and hospital clinic (Table I). One hundred and thirteen patients were treated perennially, 28 grass patients received an average of 7 injections with no unsatisfactory results, and 85 ragweed patients had 5.9 injections with 90 per cent satisfactory results. All except 2 grass patients received a top dose of 0.5 ml. to 1.0 ml. Thirty-three grass patients and 50 ragweed patients were treated preseasonally, with 5.3 and 5.5 injections, respectively, and had 93 per cent satisfactory results

Table I. *Specific treatment of pollen sensitivity with pollen protein precipitates*

Type of treatment	Pollen	No. of cases	No. of injections	Satisfactory results (%)	Final dose (ml.)*		
					(1.0)	(0.5-1.0)	(>0.5)
1958 Perennial TCA-F.T.	Grass	28	7	100	17	9	2
	Ragweed	85	5.9	90	38	47	0
Preseasonal:							
1958 TCA-F.T.	Grass	33	5.3	93	20	10	3
	Ragweed	50	5.5	87	37	11	2
1960 AC-F.T.	Grass	13	4.2	93	11	1	1
	Ragweed	35	3.8	75	32	2	1
1962 -U.F.	Grass	15	2.2	77	12	2	1
	Ragweed	33	2.3	84	26	6	1

*TCA-F.T., Trichloroacetic acid precipitate as formalinized tannate 0.25 mg. N per milliliter (25,000 PNU per milliliter).

AC-F.T., Acetic anhydride precipitate as formalinized tannate 0.25 mg. N per milliliter.

-U.F., Urea and formalin precipitate from 1-20 extract.

for grass and 87 per cent for ragweed. Fifty-seven received the top dose of 1.0 ml., 21 received between 0.5 and 1.0 ml., and 5 less than 0.5 ml.

In 1960, AC Pollen F.T. was used in the clinic to treat 13 grass and 35 ragweed patients preseasonally (Table I). The grass patients averaged 4.2 injections and the ragweed, 3.8 injections, with 93 per cent and 75 per cent satisfactory results, respectively. Forty-three patients received the top dose of 1.0 ml., 3 received between 0.5 and 1.0 ml., and 2 received less than 0.5 ml.

In 1962, Pollen U.F. was used in the clinic to treat preseasonal 15 grass and 33 ragweed patients (Table I). The grass patients averaged 2.2 injections with 77 per cent satisfactory results; the ragweed patients averaged 2.3 injections with 84 per cent satisfactory results. Thirty-eight patients received a top dose of 1.0 ml., 8 received 0.5 to 1.0 ml., and 2 less than 0.5 ml.

A separate classification of patients with no previous desensitization treatment and those treated with Pollen U.F. was made (Table II). Results of treatment are known in 45 of the 46 patients treated; satisfactory results were obtained in 42 (93 per cent).

Reactions

The local reactions varied from none to large. A typical reaction consisted of the gradual development of swelling which reached a peak diameter of 1 to 2 inches in about 24 hours, subsiding gradually after 1 to 3 days, and, if intense, leaving a nodule up to 1 cm. in size for one to several weeks. Varying degrees of "soreness" and itching were present.

In 1962, a 9-year-old boy, 3 weeks after the second injection of grass U.F., required drainage of a sterile abscess. He had grass and ragweed U.F. treatment in 1961 with no complications. (In 1961, 2 adult patients after tree U.F. injections had sterile abscesses requiring incision and drainage.) No complications occurred after F.T. injections or ragweed U.F. injections.

General reactions occurred in 8 patients on 13 occasions 1 to 12 hours after injection. Itching of the skin, with, at times, some erythema or urticaria, or mild respiratory tract symptoms "like a cold" were the usual manifestations, and subsided spontaneously or were controlled by an oral antihistamine. There were no severe acute anaphylactic reactions. It was the usual practice to permit a patient to leave immediately after treatment. No prophylactic medication was given except an oral antihistamine on rare occasions.

Table II. *Formalinized pollen precipitates in previously untreated patients*

<i>Year</i>	<i>Pollen</i>	<i>No. of cases</i>	<i>Average No. of injections</i>	<i>Satisfactory results</i>
1958	Grass	6	5.4	5 (1 ?)
TCA-F.T.	Ragweed	10	5.3	9
1960	Grass	3	4	3
Ac-F.T.	Ragweed	5	4.6	4
1961	Grass	8	2.5	8
1962	Ragweed	14	3.0	13
-U.F.				
Total		46		42/45-93%

DISCUSSION

The repository methods of current interest in the desensitization therapy of clinical allergy are the water-in-mineral-oil emulsion and the alum-precipitated pyridine-water extract. The emulsion, after extensive trial, has not received general acceptance. Alum-precipitated antigens were found to have little or no repository effect¹⁰ and other questions regarding alum-precipitated pyridine-water extracts which are soluble at alkaline pH have been discussed.¹¹ Better methods of obtaining effective repository antigens should be sought. A common method of achieving repository effects is the conversion of soluble material (e.g., insulin, penicillin) to the insoluble form. To obtain the maximum benefit from insoluble repository allergen for desensitization purposes, two conditions appear desirable or even necessary; first, conversion of the soluble allergen to a form insoluble at the alkaline pH of body fluids (± 7.4), and second, temporary blocking of the receptor groups on the allergen to inhibit immediate antigen-antibody reactions. Formalinized pollen precipitates obtained with tannic acid or urea seem to meet both requirements.

The unformalinized tannic acid precipitate from pollen extract produces typical immediate reactions on injection, intradermally, into sensitive patients. The formalinized tannate, however, produces a markedly reduced or no immediate reaction on intradermal injection; when it is injected subcutaneously, a typical pollen reaction with redness, swelling, and itching develops gradually. This observation agrees with the findings of Stull and associates¹² that formalinized ragweed extract shows reduced reactivity with specific antibody, although patients treated with formalinized extract had clinical improvement comparable to that produced by unformalinized extract. Formalin apparently blocks combining groups on the antigen inhibiting immediate reaction. Formalin also makes the protein tannate relatively insoluble at alkaline pH, resulting in slow absorption as shown by persistence of local reactions up to a week or more and by unpublished experiments with I^{131} -labeled formalinized protein tannate. With formalinized pollen precipitates, it is therefore usually possible to give a large starting dose of 2,500 PNU, and to reach a final dose of 25,000 units in 3 injections.

The question of denaturing of protein by treatment with tannic acid and formalin is often raised. Tannates of Pitressin, insulin, and bacterial toxin have been shown to be effective, and patients treated with pollen tannate showed increased tolerance to whole ragweed extract.⁴ Formalinized toxins (toxoids), are antigenically competent. That specificity is not destroyed by the chemical treatment of the pollen extract described above is indicated by the gradual typical local reactions of redness, swelling, and itching after injection into sensitive patients. Desensitization is shown by markedly reduced local reactions when a dose is repeated, and, in the course of treatment, local reactions after the larger doses are often much less than after the earlier, smaller doses. Also, skin tests to whole pollen extract are frequently smaller after treatment than before.

The clinical results obtained with the Fraction I formalinized precipitates were similar to the results with formalinized tannate derived from whole pollen

extract.⁷ By using only Fraction I and discarding fractions which do not seem necessary in treatment, some degree of purification of antigen results.

There is no significant difference between the precipitate obtained by trichloroacetic acid and acetic anhydride. It has been suggested that acetylation with other agents, such as succinic anhydride, might yield a more insoluble product. A succinic anhydride preparation was made but has not been tested sufficiently. Further work in this direction might be rewarding.

The urea formalin preparations are quite different from the formalinized tannates. Under acid conditions, formalin precipitates urea as a white insoluble resin. The original intention was to coat formalinized tannate protein particles with this resin. It became evident that the use of urea and formalin with pollen extract solutions resulted in the precipitation of protein seemingly more insoluble in alkaline pH range than the formalinized protein tannate and better tolerated. Some patients who showed a tendency to general reactions with the F.T. preparations accepted the U.F. preparation without excessive local or general reactions. When the whole pollen extract is used to make the U.F. preparation, it is uncertain which fractions of the extract are finally obtained. In 1963, only the trichloroacetic acid-precipitated pollen fraction of known N content (Fraction I) was used, and treated with smaller concentrations of urea and formalin. Clinical results in over 200 pollen cases treated in 1963 were quite satisfactory with no local or general complications and will be reported in detail later.

One solution to the problem of safe, effective repository specific treatment would seem to lie in the direction of insoluble modified antigens such as those presented above. These preparations are stable and some up to 5 years old have been used with good results. The administration is by an ordinary hypodermic injection, and the schedule may be very flexible. The use of one concentration rather than several is both more convenient and less apt to result in mistakes in dosage. The number of injections for the usual course of treatment may reasonably consist of about four injections; patients treated previously may require even fewer injections. By clinical evaluation, the results of treatment with the formalinized precipitates seem as good as or better than with other forms of treatment and appear safer to use, even by nonallergists, than other preparations available. They could be readily manufactured for commercial distribution.

For immunization purposes, the application of formalinized tannate or urea formaldehyde techniques to other antigens such as toxins and vaccines would seem promising as repositories, especially for use in humans.

It should be emphasized here that the evaluation of results such as those presented is subject to error based on bias and placebo effect. Lowell and Franklin¹³ have recently discussed the problems of setting up and evaluating double blind studies in allergic rhinitis, but their data do support the effectiveness of treatment with pollen antigen solutions. Since formalinized precipitates give clinical results apparently equal to or better than the results with conventional solutions, it is hoped that the presentation of this material will induce others to investigate such antigens by control techniques.

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