

The stability of short ragweed pollen extract as measured by skin test and antigen E

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A short ragweed pollen extract stored at 4° C was shown to retain its skin reactivity for 18 mo, although 60% of the antigen E was lost. When stored at higher temperatures, the extracts had a markedly reduced skin reactivity for some individuals, but retained full reactivity in others even though the antigen E was markedly diminished. Thus, some ragweed-sensitive individuals are very sensitive to antigen E, which is heat-labile, but some have a major sensitivity to other allergens, which are heat-stable. During the 18-mo course of this study, skin test reactions to a similar extract did not show significant variability.

Allergenic extracts are used to assist in the diagnosis and treatment of atopic diseases, but surprisingly little is known about these solutions. Investigations of certain extracts have established major active components, but parameters affecting stability have received scarce attention. Temperature and preservative are known to affect antigen E content in short ragweed pollen extract,^{1, 2} and, using skin tests³ and reagin binding techniques,⁴ similar extracts were shown to lose activity more rapidly at elevated temperatures even though their protein nitrogen content remained stable.

A number of allegedly similar commercially prepared short ragweed and grass pollen extracts were shown to differ in antigen content, skin reactivity, histamine release, and RAST inhibition activity.⁵⁻⁷ Whether such differences could result from factors such as temperature, preservative, or technical manipulation is unknown.

The present study examined the skin test reactivity of the same short ragweed extract solutions stored at 4° C for an 18-mo period. The solutions were also observed for changes in antigen E content and ability to bind reagin as measured by the radioallergosorbent test (RAST) inhibition procedure. A freeze-dried extract was employed so that the initially rehydrated and then stored solution of extract could be compared at each time interval to freshly rehydrated solutions.

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This study also permitted an examination of the variability in skin reactivity to the same allergen over an 18-mo period.

MATERIALS AND METHODS

Freeze-dried short ragweed extract (Center Laboratories, Port Washington, N. Y.). Lot No. 26907, prepared August, 1972, was reconstituted to 20,000 PNU/ml according to the directions of the manufacturer. The value redetermined at the Bureau of Biologics by Dr. Joan May was found to be 18,000 PNU/ml. Dilutions were prepared as described below.

Although earlier studies⁵⁻⁷ demonstrated that there may not be a good correlation between PNU and potency, PNU was used in this study since the vials obtained were labeled with this unitage, and the study involved only a comparison of this lot with itself under varying conditions.

The diluent was buffered physiologic saline with 0.4% phenol (Center Laboratories): the histamine solution was 1:100,000 weight/volume (w/v) calculated as free base (Center Laboratories). Injections were made with Hamilton No. 710 microliter syringes, 0.10 ml capacity, calibrated such that 0.01 ml could be delivered (Hamilton Co., Inc., Whittier, Calif.); the needles were 30-gauge, 1/2 inch (Becton, Dickinson & Co.).

Patients

Seven male and twelve female patients between the ages of 18 and 63 yr were selected from the George Washington University Hospital Allergy Clinic, where they had been followed by one of us (H. Brown) for at least 6 mo with the diagnosis of ragweed hay fever. Some of these patients were also sensitive to other allergens, and all patients were receiving hyposensitization therapy with individualized dosage schedules. The patients had not received parenteral steroid medication and were instructed not to use antihistamines for at least 24 hr prior to skin testing. An oral and written explanation of the experimental protocol was given to each patient in accord with Department of Health, Education, and Welfare (DHEW) regulations (Title 21 CFR 310.102, Paragraph H) prior to obtaining informed consent.

Procedure

The skin tests were performed according to a reproducible method suggested by the Committee on Standardization of the American College of Allergists.⁸ Briefly, a No. 30 needle on a Hamilton syringe was inserted into the superficial layers of the skin with the bevel down. Only the bevel portion of the needle entered the skin, and then 0.01 ml of the desired solution was forced into the tissue, creating a round or slightly oval wheal of approximately 4.0 mm diameter.

Freshly reconstituted solutions of ragweed extract were prepared for each day of testing and used within 12 hr of preparation. From each reconstituted vial identical series of dilutions were prepared for use on patients. For each patient dilutions were selected at the initial testing session such that large, irregular wheal formation did not occur at any concentration. The sets of solutions prepared for the first testing session became the test solution, and these solutions, which included all dilutions, were stored at 4-6° C throughout the 18-mo observation period.

Skin tests were applied 4 to 5 cm apart along a line on the lateral aspect of the upper arm. All injections were made in the same arm. After the first testing period, freshly reconstituted solutions were applied adjacent to the appropriate dilutions of the stored solution. In addition to the skin tests with extracts, each patient received skin tests with 0.01 ml of diluent and 0.01 ml of 1:100,000 histamine solution.

All skin tests were read when judged to be maximal, i.e., approximately 15 min following application. The crossed diameters of the induration and erythema were recorded, photographed, and then the sites were traced with felt-tipped pens and the tracings transferred with transparent tape to a notebook.

At the third testing period in addition to the skin tests with the appropriate dilutions of test and fresh solutions, similarly prepared concentrations of extracts were tested that had

been kept at either 24° C or 35° C. These solutions were freshly reconstituted, tested on patients, and then kept at either of the elevated temperatures for 1 wk. The solutions were then subjected to a sterility test, and during this 2-wk waiting period the solutions were maintained at 4° C.

Antigen E was measured by a radial immunodiffusion test⁹ employing a specific sheep anti-antigen E serum and using antigen E solutions, stored in 50% glycerol, as reference preparations. The antigen E was obtained from Abbott Laboratories and the antigen K from Dr. T. P. King of Rockefeller University. Each slide contained one or more concentrations of the antigen E reference in addition to the extract under test. The concentration of antigen E was determined graphically from a dose-response curve generated from 4 concentrations of antigen E varying from 5 to 40 $\mu\text{g/ml}$.

The RAST was carried out as described elsewhere,⁶ using whole ragweed extract coupled to microcrystalline cellulose. The antiserum was a pool of 5 sera from a group of untreated ragweed-sensitive individuals and is a portion of the pool previously used by Gleich and associates.⁶ The anti-IgE was prepared in rabbits against the purified Fc fragment and was labeled with ¹²⁵I. The test was carried out by incubating serum and antigen for 1 hr, adding polymer and incubating for 4 hr, and finally adding labeled anti-IgE and incubating for approximately 24 hr. The percent inhibition for each extract and antigen was determined graphically by using the average counts per minute (cpm) for the tubes with buffer as the value for 100% inhibition, and the tubes containing specific antiserum as the value for 0% inhibition.

Statistical analysis. The data in Table I were analyzed to determine whether there was any change in slope from 0 to 18 mo, and at each dose level to determine whether the stored samples were statistically different from the reconstituted samples. A comparison was made of the mean response of groups A and B to the injection of buffer alone and to histamine.

RESULTS

There is no generally accepted procedure for evaluating the results of skin testing. Thus, some⁶ determine the dilutions giving a 6 x 6 mm wheal when the buffer gives a 3 x 3 mm wheal, while others⁷ use that dilution giving a 5 to 10 mm wheal and 10 to 20 mm erythema. In our study, the buffer gave an average wheal of 4.4 mm in diameter (range of 3.6 to 5.1), and we evaluated our findings on the basis of 3 dilutions.

Skin test results, calculated as the mean of the sum of 2 crossed-diameters, revealed that the patients could be divided roughly into two groups. One group (A) responded well in the range of 1 to 10 PNU/ml (6 individuals), and the other group (B) responded in the range of 10 to 100 PNU/ml (13 individuals). In order to evaluate the results from all patients, an average value was obtained for each test strength for each individual of the two groups and recorded in Figs. 1A and 1B. The mean reaction size induced by buffer (4.3 and 4.5 mm, respectively) for groups A and B was identical, and the mean reaction sizes of all extracts stored at 4° C vs reconstituted extracts were statistically identical over the 18-mo test period. A surprising result was the comparison of histamine reactivity of groups A and B. While the wheals were identical, the mean erythema of group A, the more sensitive individuals, was larger than the mean of group B at each test period: $p < 0.01$.

The tests with extracts stored at temperatures above 4° C gave results that varied markedly with the patient; therefore, it was not possible to use average values. Table I gives the skin test results as one-half of the mean of the sum of crossed-diameters for each patient in whom extracts stored at 24° C and 35° C for 1 wk were tested. Extracts stored at 24° C for 1 wk lost little, if any, skin

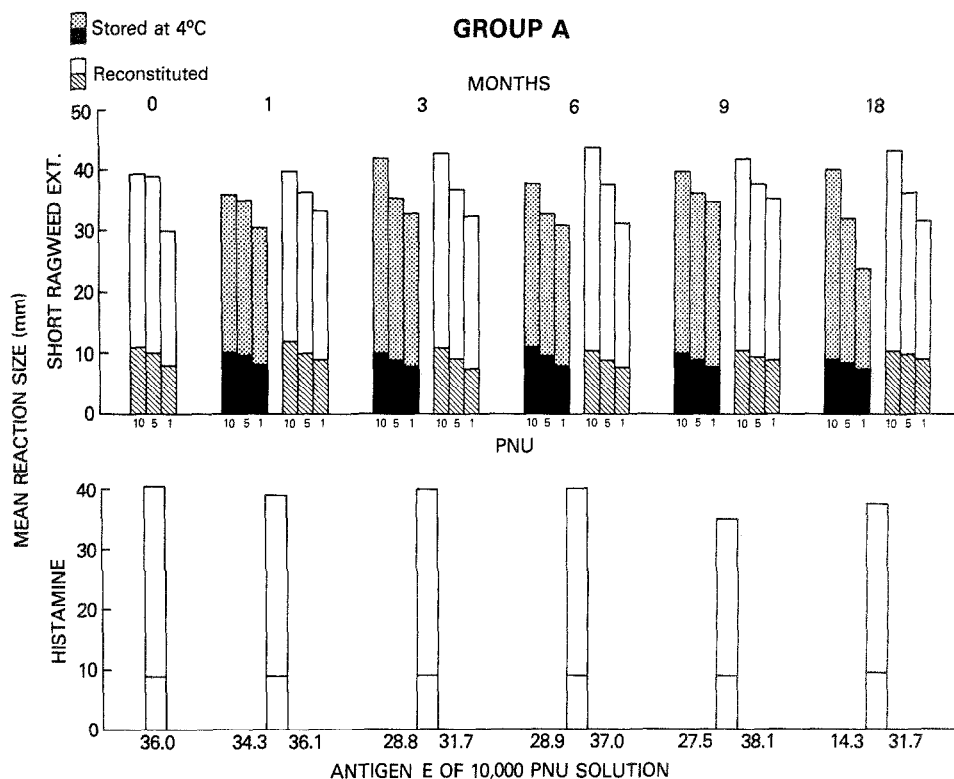


FIG. 1A. The mean skin test reactivity in two groups (A and B) of sensitive individuals to short ragweed pollen extracts stored at 4° C for an 18-mo period compared to freshly reconstituted extracts. The total length of each bar is the mean diameter of the wheal. Stored and reconstituted extracts were analyzed for antigen E content at each time period. Each individual was also tested with a histamine solution at each time interval and the mean erythema and wheal diameter recorded. Group A patients responded well in the range 1 to 10 PNU, while Group B patients were less sensitive and responded well in the range 10 to 100 PNU.

reactivity. However, in two individuals, 7 and 9, a marked diminution in reactivity was evident. On the other hand, extracts stored at 35° C were either unchanged or showed only slight diminution in reactivity in some individuals,^{10, 12, 13, 15-18} especially at higher dilutions, while exhibiting a striking loss in reactivity in others.^{11, 14} Both small and large differences in reactivity were seen in groups A and B.

As seen from Figs. 1A and 1B, the antigen E content of the freeze-dried extract did not change over an 18-mo test period. When freshly reconstituted to 10,000 PNU/ml at each time interval, the antigen E content varied between 31.5 and 38.1 µg/ml. The extract stored at 4° C showed a steady decline in antigen E to approximately 40% of the freshly reconstituted material.

Table II gives the antigen E content and capacity to inhibit in the RAST for a set of extracts stored for various time periods and at 4° C, 24° C, and 35° C.

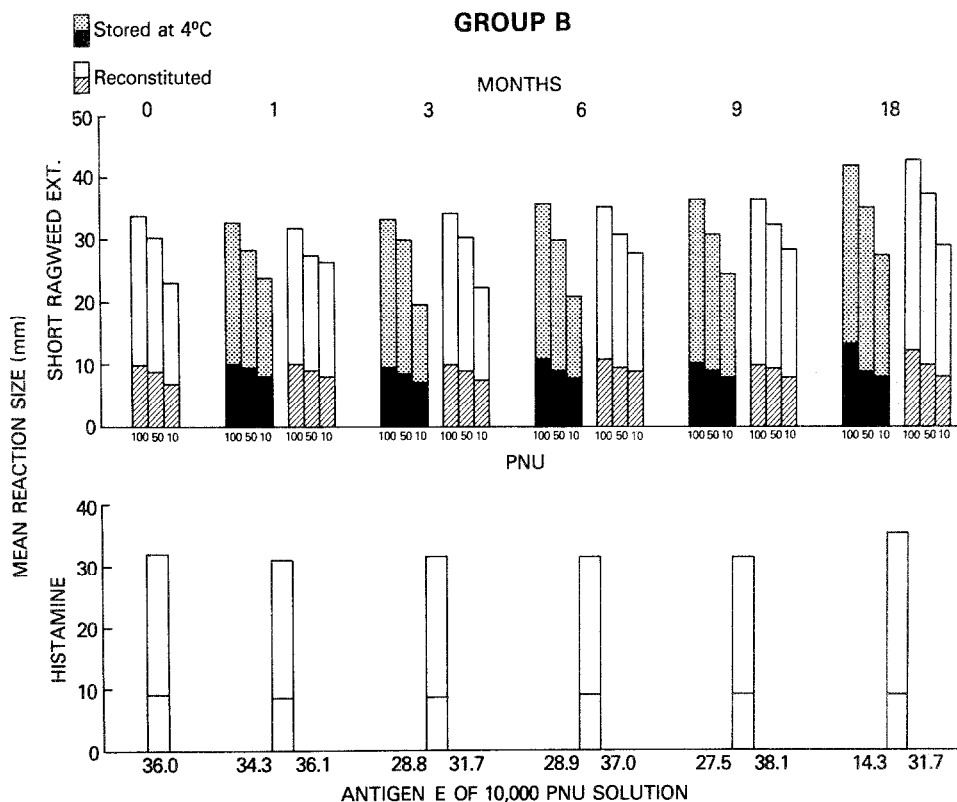


FIG. 1B. For legend, see Fig. 1A.

It can be seen that the antigen E content, as measured by radial immunodiffusion in agar gel, declines more rapidly than the reagin binding capacity. This is shown clearly in the ratio of percent inhibition to antigen E content. As the extracts age, the ratio gets larger. For the 10,000 PNU extract maintained at 4° C, the ratio changed from 4.7 for a 9-month-old extract to 6.5 for the 27-month-old extract. The ratios get much larger in the extracts maintained for 1 wk at 24° C and 35° C. Thus, a fresh extract of 20,000 PNU with a ratio of 1.4 changed to 5.2 after 1 wk at 24° C, and to 17.1 after 1 wk at 35° C. The increased temperature of 35° C caused a rapid loss of precipitability in agar, but only a slow loss in ability to bind to reagin.

To help in evaluating the RAST inhibition, pure antigens E and K were compared to a whole short ragweed pollen extract (Table III). Antigen E was capable of inhibiting 72% of the reagin in the pooled serum, while antigen K inhibited 64%. The two antigens together were no more effective in inhibiting than they were individually. Whole short ragweed pollen extract, however, gave complete inhibition. Thus, other allergens are present in whole extract that can combine with reagin. It is also striking that whole extract containing 1.4 μ g of antigen E neutralized almost all of the reagin, while 10 μ g of pure antigen E was not as effective.

TABLE I. Effect of storage at 24° and 35° C for 1 wk on the skin reactivity of short ragweed pollen extract

Group	Test dose (PNU)	Patient											
		1		2		3							
		4°	24°	4°	24°	4°	24°						
A	10 W*	9.5	10.0	14.0	9.0	12.0	10.0						
	E	42.5	35.5	36.5	34.0	47.5	42.5						
	5 W	8.0	7.5	12.0	9.5	10.0	7.0						
	E	32.0	34.5	33.5	32.5	42.0	38.5						
	1 W	7.5	7.5	7.5	8.5	9.0	8.0						
	E	27.0	28.0	30.0	27.5	40.0	42.5						
B	100 W	12.5	13.0	14.5	14.5	17.5	19.5	8.0	4.5	8.0	9.0	8.5	8.5
		E	41.0	45.0	35.0	36.0	36.0	38.0	30.5	16.5	34.0	32.5	35.0
	50 W	10.5	10.5	13.0	14.5	17.0	12.0	7.0	6.5	5.5	5.5	8.5	7.5
		E	36.5	40.0	40.0	35.0	32.5	31.5	26.0	24.0	14.5	12.5	34.0
	10 W	9.0	9.0	12.5	12.0	11.0	11.5	6.5	6.0	5.5	5.5	8.5	6.5
		E	32.5	37.5	40.0	35.0	21.0	27.0	29.0	14.0	0	0	33.0

Antigen E content of 10,000 PNU solution $\mu\text{g/ml}$ †

35.1 28.7

*The numbers represent the mean of 2 diameters of wheal (W) and erythema (E). Patients 1 to 9 were tested on the same arm with dilutions of extracts stored for 1 wk at 4° and 24° C; Patients 10 to 19 were similarly tested with extracts stored at 4° and 35° C.

†This is the material from which dilutions were prepared. The stored material for skin testing was diluted at time 0. Material for antigen E analysis was stored as the 10,000 PNU solution at the appropriate temperature.

TABLE II. Antigen E content and reactivity, as measured by RAST inhibition, of a short ragweed pollen extract stored at 4, 24, and 35° C for varying periods of time

Concentration* of extract (PNU)	Time stored (mo)	Storage temperature (° C)	Antigen E content ($\mu\text{g/ml}$)	% inhibition using 0.1 ml	% inhibition antigen E
10,000	9	4°	16.0	75	4.7
	18		11.3	71	6.3
	21		9.8	57	5.8
	24		9.8	65	6.6
	26		9.3	63	6.8
	27		10.2	66	6.5
20,000	0	4°	63.0	86	1.4
	9		35.4	91	2.6
	18		18.6	83	4.5
	21		19.1	80	4.2
	24		14.1	80	5.7
	26		14.1	70	5.0
	27		18.2	76	4.2
20,000	1/4	24°	13.5	70	5.2
	1/4	35°	3.4	45	13.2
	1/4	35°	2.8	48	17.1

*These are not the extracts from which skin test dilutions were made although they were prepared at the same time.

number													
10		11		12									
4°	35°	4°	35°	4°	35°								
9.4	10.0	8.0	8.5	8.5	8.0								
37.5	37.5	18.5	0.0	33.0	40.0								
9.5	6.5	9.0	5.5	8.0	7.5								
40.0	31.0	16.5	0.0	40.0	31.5								
8.5	6.0	7.0	5.5	8.5	6.0								
37.5	27.0	12.5	0.0	33.0	14.0								
13		14		15		16		17		18		19	
4°	35°	4°	35°	4°	35°	4°	35°	4°	35°	4°	35°	4°	35°
7.5	7.0	7.0	7.5	14.0	12.0	13.0	7.5	10.0	8.0	7.5	7.5	7.0	6.5
40.0	34.5	35.0	23.5	33.5	33.5	36.5	26.0	31.5	31.5	37.5	37.5	17.5	9.5
8.5	5.5	8.0	5.0	13.0	10.5	12.0	6.5	10.5	7.5	8.0	5.0	7.0	5.0
33.0	27.0	30.0	0.0	27.5	25.0	30.5	30.5	34.0	31.0	36.5	30.0	0.0	0.0
7.5	4.5	5.5	5.5	10.0	14.0	6.0	3.5	7.5	6.0	6.5	6.0	4.5	4.5
28.0	8.0	23.0	0.0	24.5	22.5	31.5	0.0	19.5	10.0	29.0	0.0	0.0	0.0
35.1	9.1												

DISCUSSION

The division of the individuals into two groups, Group A being very sensitive and Group B being less sensitive, demonstrated that the degree of sensitivity of test subjects did not appear to be a factor in this study. Although these individuals were under treatment, it is known that IgG antibodies do not affect the binding of IgE to antigen.¹⁰ Since all of the individuals were undergoing hyposensitization treatment, consideration may be given to the hypothetical possibility that the material injected may induce IgE to some stable nonallergenic components of the extract. However, there is no evidence that this occurs.

The mean skin-reactivity was constant throughout the 18-mo observation period, since the slopes of the skin responses vs time were not statistically different from zero. IgE levels do change with season,¹⁰ but the variation is apparently not large enough to have a marked effect on skin reactivity.

The mean skin reactivity of all dilutions of stored extracts (at 4° C) and freshly reconstituted extracts was identical. By this criterion, there is no loss of reactivity of the stored extracts over the 18-mo test period. On the other hand, approximately 60% of the antigen E had been lost as measured by an agar gel diffusion test (Figs. 1A and 1B). The failure of antigen E content to correlate with skin reactivity for this time period may be due to several causes. Antigens other than antigen E may be more stable at 4° C. Also, the ability to precipitate with antibody in agar gel diffusion may be lost more rapidly than the antigen's ability to bind to specific IgE antibodies. Evidence for this second point, demonstrated by the ratio of percent inhibition to antigen E, is seen in Table II. There thus appears to be a sequence of events in which precipitability of antigen E dis-

TABLE III. Inhibition of reactivity of short ragweed pollen extract by antigens E and K as measured by RAST inhibition

Antigen added (μ g)*		Storage temperature ($^{\circ}$ C)	Storage time (days)	Percent inhibition
E	K			
10		4		72
5				65
1				48
0.1				41
5		35	3	59
1				47
0.1				35
5		35	5	31
1				0
0.1				0
	10	4		64
	5			51
	1			23
	0.1			8
	5	35	3	30
	2			2
	1			0
	5	35	5	0
	2			0
	1			0
10	10	4		76
5	5			75
2.5	2.5			53
1	1			43
Whole ragweed extract				
14†				100
1.4				91
0.14				73
0.014				32

* μ g of pure antigen E or K added.† μ g of antigen E in the whole ragweed extract.

appears before binding capacity. This could provide an explanation of Figs. 1A and 1B where skin reactivity, which depends on binding capacity, appears to remain unchanged over the 18-mo storage period while antigen E, measured as the ability of antigen E to precipitate with a sheep antiserum, declines by 40%. Apparently both types of binding eventually can be aborted. For instance, skin reactivity of ragweed extracts will diminish after years, even at refrigerator temperature.³ As seen in Table III, when antigen E is warmed to 35 $^{\circ}$ C for 7 days, both precipitability and binding power are lost, and this probably represents an acceleration of what occurs at 4 $^{\circ}$ C over a period of years.

Storage of six dilutions of the extract at 24 $^{\circ}$ C and 35 $^{\circ}$ C produced unanticipated results. Heating to 56 $^{\circ}$ C has been shown to rapidly destroy skin reactivity.³ The temperatures used in this study, 24 $^{\circ}$ C and 35 $^{\circ}$ C, resulted in loss in activity that was dependent on the individual in whom the test was carried out. This clearly demonstrates that antigen E (and/or other labile antigen, such as antigen K) is probably the major antigen for some individuals, but other more heat-stable antigens may be dominant in other ragweed-sensitive individuals. The

importance of other antigens has been established in other instances. For instance, Prausnitz-Küstner sites exhausted by antigen E were still very reactive to whole extract,¹¹ and fractions of short ragweed pollen extract devoid of antigen E were very skin-reactive.¹²

Nevertheless, the fact that antigen E may bind a major portion of the reagin and demonstrate skin reactivity in 90% of ragweed-sensitive individuals¹³ establishes antigen E as an important allergenic component of short ragweed pollen extracts. The heat lability of antigens E and K suggests that they are very valuable and sensitive standards of the quality of short ragweed pollen extracts. Finally, it demonstrates the need for large pools of sera if the RAST inhibition is to be used for the potency assay of extracts, so that all allergenic determinants will be included and will lead to general reproducibility of results.

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