

The heat stability of short ragweed pollen extract and the importance of individual allergens in skin reactivity

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A short ragweed pollen extract that was heated in a boiling-water bath remained skin reactive for some allergic individuals and lost reactivity for others. Comparison of heated and unheated extract by immunodiffusion showed that AgE, AgK, and Ra3 could not be detected in the boiled extract but Ra5 was present. By inhibition of the radioallergosorbent test the concentration of Ra5 remained essentially unchanged, but the concentrations of AgE and Ra3 were greatly diminished. The sera of these ragweed-allergic individuals were analyzed for IgE antibodies to AgE, Ra3, and Ra5. Skin reactivity in 17 ragweed-allergic individuals to the heated extract correlated significantly with the levels of IgE antibodies to Ra5. However, the large number of individuals reacting to the boiled extract indicates that Ra5 is not likely to be the only heat-stable allergen. Skin reactivity of the unheated extract did not correlate with IgE antibody levels to any allergen. The results underscore the importance of individual allergens in the materials used for skin testing.

Several of the proteins that have been identified and characterized in extracts of short ragweed pollen exhibit allergic activity.¹ These allergens—AgE, AgK, Ra3, BPA-R (Ra4), and Ra5—have been well studied, and other allergens undoubtedly exist.^{2, 3} AgE is reactive at very low concentrations in the skin of almost all individuals who are allergic to ragweed,⁴ and is also important for immunotherapy.⁵ Although the other allergens are skin reactive in certain ragweed-allergic individuals, their importance to the reactivity of whole short ragweed pollen extract has not been well explored. One study found that skin reactivity did not correlate with the AgE content of fractions of short ragweed extract, thereby suggesting the importance of other allergens.⁶ A short ragweed extract heated to 35° C showed a large decrease in AgE content as measured by radial immunodiffusion (RID)

and while skin reactivity to the heated extract was markedly reduced in some individuals, only a minimal change was observed in other patients.⁷ Using the radioallergosorbent test (RAST), a short ragweed extract produced inhibition reactions at concentrations that could not be attributed solely to AgE content.⁷ All of these studies suggest that allergens other than AgE play a significant role in ragweed allergy.

Because it was found by one of us (R. H.) that a short ragweed extract could be heated in boiling water without reducing skin reactivity in certain allergic individuals, this study examines the role of allergens other than the known heat-labile AgE and AgK.

MATERIALS AND METHODS

Preparation of extracts

Seven grams of short ragweed pollen (*Ambrosia elatior*) were extracted in 70 ml of Coca's solution for 4 hr at room temperature and then sterile filtered. Two 10-ml aliquots were diluted with equal volumes of glycerine and one of these was placed in a boiling water bath for 1 hr.

Analysis of pollen extracts

The content of AgE was determined by RID.⁸ Specific antisera for AgK, Ra3, and Ra5 (the latter obtained from Dr. David Marsh) were used to detect the presence of these allergens in the boiled and unboiled preparations using the Ouchterlony technique. The relative concentrations of AgE,

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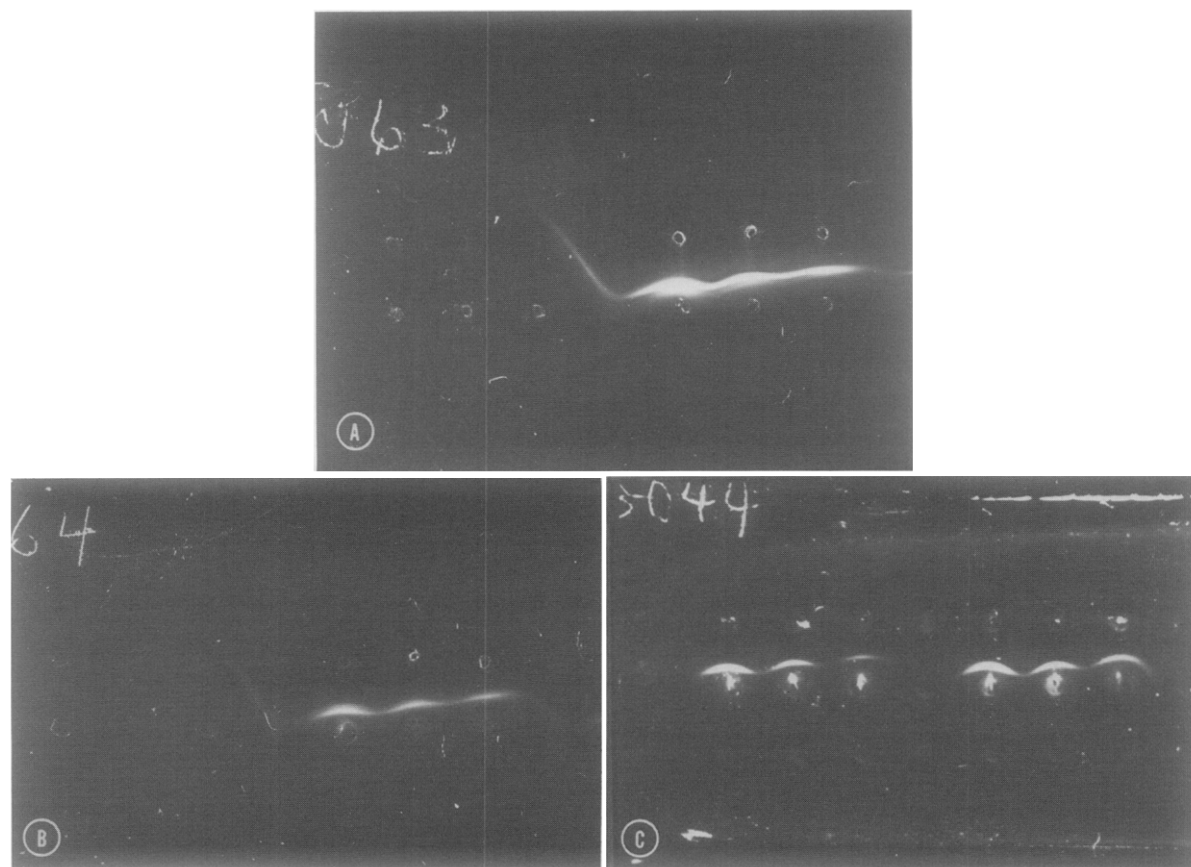


FIG. 1. Gel diffusion of a 1:10 short ragweed pollen extract before and after heating. *A*, *B*, and *C*: The six lower wells shown in each photograph contain antiserum: in *A*, anti-AgE; in *B*, anti-AgK, and in *C*, anti-Ra5. The three upper left wells of each photograph contain boiled extract at (*A*) 1:10, (*B*) 1:20, and (*C*) 1:40. The three upper right wells contain unboiled extract at (*A*) 1:10, (*B*) 1:20, and (*C*) 1:40.

Ra3, and Ra5 were measured by RAST inhibition using supports coupled with these allergens. Antibodies to AgK were not studied quantitatively because of the high degree of cross-reactivity between AgE and AgK.⁹

Radioallergosorbent test (RAST)

The solid-phase materials—AgE, Ra3, and Ra5—used in the RAST have been described previously.⁹ In the performance of the RAST we used a modification of a procedure described elsewhere.^{10, 11} Briefly, 0.05 ml of a 1.0-mg/ml cellulose suspension was added to each tube, followed by 0.15 ml of RAST diluent (0.1 M KH_2PO_4 – K_2HPO_4 [pH 7.4], 0.2% bovine serum albumin [BSA], 1% Tween 20, and 0.1% sodium azide) and 0.05 ml of serum. After overnight rotation (not end-over-end tumbling) and two washing steps with 1.0 ml of RAST wash (0.1 M KH_2PO_4 – K_2HPO_4 [pH 7.4], 1% Tween 20), 5 ng of anti-IgE in 0.1 ml of 0.1 M KH_2PO_4 – K_2HPO_4 [pH 7.4] with 1% BSA and 0.150 ml of fetal calf serum (FCS) diluent (same composition as RAST diluent, except FCS 4% is substituted for the BSA) were added to each tube. The tubes were again rotated overnight and washed twice the following day be-

fore the radioactivity associated with the solid-phase allergen was determined on a gamma spectrometer. The results, given in arbitrary RAST units, were determined by interpolating the percent counts bound from each serum on a dose-response curve obtained by utilizing dilutions of a serum pool from five ragweed-sensitive patients.

Skin testing

The individuals used in this study were 59 unselected ragweed-allergic individuals who exhibited large scratch-test reactions on initial testing with ragweed extract. Of the 59 patients, 34% reacted only to unboiled extract, 17% reacted to unboiled with a slight reaction to boiled extract, 34% gave only a slightly larger reaction to unboiled than boiled extract, and in 15% there was no difference in reactivity to unboiled and boiled extract. On the basis of the initial scratch test, 17 individuals representing each of the above were selected for the more detailed study reported in this paper. In eight individuals, the extracts were of approximately equal skin reactivity (patients 2, 4, 5, 7, 8, 9, 10, and 11); in two, reactivity to the unheated was slightly greater than to the boiled extract (1 and 6); in five, reactivity

to the unheated was much more reactive than to the boiled extract (3, 12, 13, 14, and 15); and two reacted only to unheated extract (16 and 17).

Total IgE

Total IgE was measured by the paper radioimmunosorbent test (PRIST), using kits purchased from Pharmacia Laboratories (Piscataway, N. J.).

RESULTS

The short ragweed pollen extract contained 225 $\mu\text{g/ml}$ AgE by RID. Analysis by Ouchterlony (Fig. 1,A) showed two bands for AgE that appear to merge when the antigen is diluted (the cause of this double banding is not certain). After boiling, there was no detectable AgE by RID. The minimum detectable AgE by this procedure is 5 $\mu\text{g/ml}$. Heating also destroyed AgK (Fig. 1,B) and Ra3 (not shown). Figure 1,C shows, for three dilutions of the extract, that there is little difference between the boiled and unboiled extracts when antiserum for Ra5 is used. Loss of ability to precipitate with rabbit antibody in gel does not necessarily mean that all binding capacity of an antigen or the ability to react with IgE antibody is destroyed. Therefore, we determined whether boiled extracts could inhibit in the RAST using purified AgE, Ra3, and Ra5 as solid-phase allergens. By this test, the boiled extract had less than 0.05% of the original AgE and only 0.8% of the original Ra3. However, there was no detectable loss of Ra5 after boiling (Table I).

A study was conducted of the reproducibility of the boiling phenomenon. In the absence of glycerol, heavy precipitation is usually observed. In the presence of glycerol, the boiled extract may be clear or slightly turbid and AgE, AgK, and Ra3 are almost undetectable while Ra5 remains almost intact. The heating process was studied by placing extracts in a boiling water bath for 15 min or 1 hr. Extracts were prepared with and without 50% glycerol from three pollen lots collected in two different years. In five trials, heating for 15 min with or without glycerol left the Ra5 detectable. Heating for 1 hr without glycerol resulted in loss of Ra5 in four trials. Heating for 1 hr with 50% glycerol resulted in loss of Ra5 once in five trials.

Table II shows the sums of the crossed diameters of wheals produced by unheated and boiled ragweed extract in order of decreasing sizes, together with the specific IgE antibody titers expressed as RAST units for antibodies AgE, Ra3, and Ra5, as well as total IgE and the previous immunotherapy status of the 17 individuals studied. The rank-order correlation coefficient between levels of IgE antibody and skin reactivity

TABLE I. RAST inhibition with solid-phase purified allergens

	Solid phase extract (μl for 50% inhibition)*		
	AgE	Ra3	Ra5
Glycerinated short ragweed extract (1:20 dilution)	0.052	1.04	0.021
Glycerinated short ragweed extract boiled 60 min (1:20 dilution)	>100†	138.88	0.019

*Volumes computed from regression line.

†Fifty percent inhibition was not reached with the maximum test volume of 100 μl .

is not significant for any allergen other than Ra5 and the skin reactivity to the boiled extract. For this allergen, the correlation between reaction size and antibody levels to Ra5 is significant at $p < 0.01$. The results were also tested using only sera in which the levels of IgE antibodies to Ra5 were clearly elevated, namely sera from patients 2, 3, 4, 7, 9, 11, and 13. The skin reactivity to boiled extract in these patients was significantly correlated with the elevated levels of IgE antibodies to Ra5 ($r_s = +0.81$; $p < 0.05$). There was no relationship to total IgE or to previous immunotherapy; the three highest values for anti-Ra5 were from nontreated individuals, while several of the lowest were also in this group. If the individuals are arranged in decreasing size of their skin reactions to the unheated extract, there is no correlation between reaction size and any of the other parameters. In carrying out rank-order analyses, background values were not subtracted.

DISCUSSION

There is little doubt that AgE is one of the most important allergens responsible for short ragweed allergy, largely based on the fact that almost all short ragweed-allergic individuals react to it by skin test in very low concentrations, the majority reacting at between 10^{-10} to 10^{-12} gm, and some reacting even at 10^{-14} gm,⁴ and most have specific IgE to it in their sera.⁹ However, it has been claimed that extracts or fractionated extracts with little or no AgE can be intensely skin reactive.⁶ Consequently, it seems likely that allergens other than AgE play a role in a significant number of ragweed-allergic patients.

Our finding that boiling a short ragweed extract may, for some individuals, have a negligible effect on its ability to react in the skin further emphasizes the role of multiple allergens in sensitivity to short ragweed pollen. The data show that there are heat-stable

TABLE II. Correlation of skin reactivity of ragweed-sensitive individuals with IgE titers

Patient	Boiled short ragweed extract (wheal mm)*	Unheated short ragweed extract (wheal mm)*	IgE antibody titers			Total IgE (IU)	Therapy
			AgE †	Ra3 †	Ra5 †		
1	42	56	102.0	3.8	3.6	496	—
2	40	43	49.2	0.6	170.0	103.5	—‡
3	40	66	44.4	3.9	18.0	151	+
4	39	39	391.5	189.2	30.6	425	—
5	36	35	10.5	3.9	3.8	<1	+
6	35	42	2.4	2.9	2.3	4	—
7	34	34	0.8	0.0	20.8	29	+
8	32	29	9.6	3.2	3.0	120	§
9	27	33	5.6	5.7	6.6	227	+
10	23	29	2.8	1.5	0.9	<1	+
11	22	21	20.0	7.5	7.4	22	—
12	10	44	81.4	14.3	1.5	64	—
13	9	26	54.5	2.5	5.3	453	+
14	8	41	29.2	22.9	1.6	73	—
15	4	33	3.8	0.0	0.0	250	§
16	0	21	13.6	2.8	1.7	346	—
17	0	52	6.4	3.0	1.7	21	—
Average normal controls			1.2	0.8	2.4		

Rank correlation, boiled extract: Wheal vs anti-AgE = 0.263, NS; Wheal vs anti-Ra3 = 0.118, N.S.; Wheal vs anti-Ra5 = 0.646, $p < 0.01$. Rank correlation, unheated extract: Wheal vs anti-AgE = 0.298, NS; Wheal vs anti-Ra3 = 0.225, NS; Wheal vs anti-Ra5 = 0.097, NS.

*Sum of crossed diameters.

†Arbitrary RAST units.

‡Last therapy 20 yr ago.

§It was not certain that these individuals had immunotherapy with short ragweed in the past.

||Last therapy 9 yr ago.

and heat-unstable components in short ragweed extract. As is well known, AgE and AgK are heat labile and Ra3 also appears to be heat labile. Ra5 is heat stable; specific antisera were not available to test for Ra4 or the more recently discovered allergens.² Ra5 has many disulfide bonds and is also unique because of its very low molecular weight, approximately 5,000. Also, when short ragweed pollen is extracted, Ra5 appears very rapidly in the extract; in fact, it is completely extracted from the pollen when there is very little AgE in the extract.¹ The current finding that the presence of specific IgE to Ra5 is the only antibody that correlates with skin reactivity to boiled short ragweed extract is added evidence of its importance. However, the number of individuals reacting to the boiled extract is greater than the number expected to react with Ra5; consequently, other heat-stable allergens are also likely present. While this finding does not alter the importance of AgE, it suggests that all allergens in short ragweed pollen are required for accurate diagnosis and presumably also for immunotherapy. Therefore, the use of extracts from which "extraneous components" have been removed by

various fractionation processes must be viewed with caution. Extracts deficient in one or more components may still show a statistically significant effect when used for immunotherapy. However, such extracts may be ineffective in diagnosis and in therapy for individuals with high levels of IgE antibody to particular components of the mixture.

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