

ANTIGENS OF *AMBROSIA ELATIOR* (SHORT RAGWEED) POLLEN

II. Immunochemical Identification of Known Antigens by Quantitative Immuno-electrophoresis¹

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Previously isolated allergens AgE, AgK, Ra5, and Ra6 of short ragweed pollen were identified as antigenic components within an established crossed immunoelectrophoretic (CIE) ragweed-anti-ragweed reference system. On the other hand, antigen Ra3 could not be identified because of the very low concentration of anti-Ra3 in the anti-ragweed antibodies used and the unfavorable pI values of Ra3 and anti-Ra3. All antigens except 1 preparation of Ra5 contained detectable impurities, but in most cases only in trace amounts, as estimated by mathematical iteration of the CIE precipitates. The previously established partial immunologic identity between AgE and AgK was verified, and it was found that 2 further antigens were included in this cross-reacting complex. The usefulness of CIE techniques in antigenic analysis and identification is clear from these studies.

In the previous paper of this series (1), it was shown by CIE that short ragweed pollen extract consists of a complex array of at least 52 antigenic components. We now investigate which of the antigens in our CIE reference pattern correspond to the defined allergens, antigen E (AgE)³, antigen K (AgK), Ra3, Ra5, and Ra6 (2-8)⁴. In particular, we report studies of the antigenic components of the AgE-AgK cross-reacting complex (4).

MATERIALS AND METHODS

Crude ragweed antigens and anti-ragweed antibodies. Dialyzed 20-hr (5°C) extracts of short ragweed pollen (*Ambrosia elatior*), Am.e.76, Am.e.78, and Am.e.80, and homologous rabbit anti-ragweed antibodies were prepared as described previously (1). A dialyzed 16-min extract of short ragweed pollen and a pooled rabbit antibody, anti-Am.e.(16-min), were prepared as described elsewhere (8).

Purified antigens. Purified electrophoretic components of AgE, IVA, IVB, and IVC, and partially purified AgE-IVD and AgK-A, were prepared by

ammonium sulfate fractionation of defatted short ragweed pollen extract followed by chromatography on DEAE-cellulose (9). By polyacrylamide gel electrophoresis (PAGE) at pH 9.5, the samples of AgE-IVA, IVB, and IVC showed the presence of contaminants only when the gels were overloaded (>10 µg per disc gel), whereas the samples of AgE-IVD and AgK-A both showed more than 1 component when the gels were loaded with less than 10 µg per gel. A further AgE preparation, AgE-NIH, was obtained from the Research Resources Branch, NIAID, NIH, through the courtesy of Dr. Daniel Mullally. This material gave rise to 4 protein-staining bands on agarose electrophoresis at pH 8.6 (10), and 2 major and 1 minor band on SDS-PAGE (corresponding in m.w. to the dissociated α and β chains of AgE and a small amount of undissociated material).

A highly purified Ra3 fraction (denoted Ra3-1) was prepared using, as starting material, ragweed Fraction C (2; M. Roebber and D. G. Marsh, unpublished observations). In brief, the method consisted of ultrafiltration through a series of Amicon hollow fiber cartridges. The Ra3-containing fraction was purified by column chromatography on QAE-Sephadex and Sephadex G50 (superfine) with procedures similar to those previously described (6) and, finally, by chromatography on a CM-Sephadex column from which the Ra3 was eluted with 0.005 M phosphate at pH 7.0. The resultant material gave single bands on agarose gel electrophoresis at pH 7.8 and isoelectric focusing (pH 3.5 to 9.5) in acrylamide gel. Two components were found by SDS-PAGE that corresponded to the 2 forms of Ra3 (6). The Ra3-1 preparation had an N-terminal amino acid sequence (12 amino acid residues) identical to that recently described by Klapper *et al.* (11); D. G. Klapper, personal communication. Antigen Ra3-NIH was obtained as a Research Reference Reagent from the NIH. This fraction was found to contain 2 major and 1 minor protein-staining bands on agarose electrophoresis at pH 8.6 (10) and 2 bands on SDS-PAGE.

Highly purified antigen Ra5 (denoted Ra5-1) was prepared according to the procedure of Roebber *et al.* (12). A partially purified preparation of Ra5, Ra5-2 was obtained as a side-fraction during the purification of Ra6⁴. Data are included in order to emphasize the resolving power of CIE techniques. Antigen Ra5-3 was prepared according to Lapkoff and Goodfriend (7) with modifications, including high-voltage paper electrophoresis as a final step (L. Goodfriend, unpublished). Both Ra5-1 and Ra5-3 gave single bands on PAGE at pH 4.3 and SDS-PAGE, and both gave single precipitin arcs on immunodiffusion and immunoelectrophoresis against goat anti-Ra5 and a pool of rabbit anti-whole ragweed antibodies.

Allergen Ra6 was prepared as described by Hussain and Marsh⁴. This material gave 1 band on PAGE at pH 4.3 and 1 band on SDS-PAGE. One major and 2 trace minor antigens were detected by CIE against potent anti-Am.e. (16-min) serum pool. A partially purified fraction, Ra6-X (8), which contained Ra6 and an unidentified antigen, was also studied.

Antibodies. Goat antibodies were produced against a previously prepared highly purified Ra5 antigen (8). Goat anti-Ra3(NIH) was prepared by repeated immunization with Ra3-NIH (1 mg) emulsified with Freund's complete adjuvant containing 10 mg/ml of *M. butyricum* (10 times the usual amount).

Electrophoretic analyses. Quantitative immunoelectrophoretic (QIE) analyses, including CIE, CIE with an intermediate gel, and crossed-line immunoelectrophoresis (CLIE), were performed as described in a recent review (13) and the previous paper in this series (1). Unless otherwise indicated: a) The agarose gels were 1.5, 1.2, and 1.0 mm thick for first, intermediate, and second dimension gels, respectively. b) The temperature of the cooling water was 15°C. c) The first and second dimension electrophoresis experiments were carried out at pH 8.6 for 25 min and 16 hr, respectively. In order to improve the resolution of immunoprecipitates in experiments performed using Ra3 and Ra5, it was necessary to run first and second dimension gels on the same plate rather than the usual procedure of transferring the first dimension gel to a second plate (1). The amounts of

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³ Abbreviations used in this paper: Ag, antigen; Am.e.76, Am.e.78, and Am.e.80, 1976, 1978, and 1980 collections of the pollen of *Ambrosia elatior* (short ragweed); QIE, quantitative immunoelectrophoresis; CIE, crossed immunoelectrophoresis; CLIE, crossed line immunoelectrophoresis; CRIE, crossed radioimmunoelectrophoresis.

⁴ R. Hussain and D. G. Marsh. Rapidly released allergens from short ragweed pollen. III. Purification, characterization and allergenic activity of a new allergen - Ra6. In preparation.

antigenic impurities in the purified allergen preparations were obtained by mathematical iteration (repeated approximation until a reliable result is obtained). This was performed on the basis of: a) the weight amounts of the purified allergen and reference extract used in QIE experiments; b) the relative amounts of allergen in the purified fraction and reference determined by integrating the areas under the precipitates by means of QIE (13); c) calculation of the percent contribution from unknown antigens in the reference; d) assuming a linear correlation between titer and area for all unknown antigenic impurities as a first estimation. The results of this first approximation in the iteration were included in the next step and repeated until successive computed answers were within $\pm 5\%$ of one another. For example, 2.05 μg AgE-IVA gives rise to the same Ag 10E delimited precipitation area as 140 μg Am.e.76 against the same antibodies, by means of QIE. The integrated areas of the impurities (= non-Ag 10E) in these 2 samples are 49 and 146,400 arbitrary units, respectively. The impurities in AgE-IVA thus correspond to:

$$\frac{137.95 \times 49}{146,400} = 0.046168 \mu\text{g}$$

Assuming that 2.003832 μg of the AgE-IVA is Ag 10E and repeating the calculation will give 0.046184 μg impurities, which is also the result of the third calculation. The antigenic impurities thus have been estimated to be 2.25%, corresponding to 98% Ag 10E in AgE-IVA.

RESULTS

The designation of ragweed antigens was according to the CIE reference pattern described in the preceding paper of this series (1), which is reproduced as Figure 2B.

Analysis of the AgE and AgK preparations. Figures 1A–D show the CIE patterns for the 4 isolated isoallergenic components of AgE against anti-Am.e.76, and Figure 1E shows the corresponding pattern for AgE-NIH. These samples all contain 1 common major component (AgE) and other minor components in varying amounts. Integration of the areas under the precipitates of the different components, followed by iteration, suggests that these samples of AgE-A, -B, -C, -D, and -NIH contain about 2, 2, 5, 37, and 10% of impurities, respectively, several of which could be identified as designated antigens in the CIE reference pattern (Table I). These levels of impurities are in accord with the qualitative findings on disc PAGE. As expected, AgE-A, -B, -C, and -D exhibit increasing electrophoretic mobilities at pH 8.6. Inspection of the agarose electrophoretic pattern of AgE-NIH indicates that the major components are the B, C, and D forms of AgE. Analysis of AgK-A (Fig. 1F) showed that the partially purified fraction consisted of a major component together with several impurities comprising about 7% of the fraction (Table I).

Antigens E and K were identified as Ags 10E and 10K, respectively. Examples of CLIE experiments illustrating these results for AgE-NIH and AgK-A are shown in Figure 2. Antigen 10E showed a reaction of partial immunologic identity with 3 other antigens, 10K, 11, and 27a, which were all present in most of the AgE and AgK preparations. The relationship between AgE (10E) and AgK (10K) can be seen from the fusion of curved and line precipitates indicated by arrows in Figures 2C and D and comparison of these precipitates with the CIE reference pattern (Figs. 2A and B). The identification of Ags 11 and 27a and the cross-reactions between these antigens and Ags 10E and 10K were apparent from careful examination of these and other CLIE experiments. Comparison of fraction AgE-IVB with AgK-A by means of CIE and CLIE (Figs. 3A and B) showed that the major antigen of AgE (10E) was antigenically related to AgK. This is clear from the CLIE (Fig. 3B), where antibodies against AgE (10E) were only partially absorbed by AgK. In similar experiments (Figs. 3C and D), AgK (10K) was found to be partially identical to AgE. In both CLIE experiments, line precipitates fused with curved precipitates, which showed that the AgE and AgK preparations contained identical epitopes.

Analysis of Ra3. Antigen Ra3 proved to be the most difficult antigen to analyze because: a) The isoelectric points of the components in the 2 preparations were close to pH 8.6, the pH of the running buffer. b) The isoelectric points of homologous antibodies were also in the region of pH 8.6. c) The anti-crude ragweed antisera contained almost no anti-Ra3 (traces were detectable by radial immunodiffusion experiments). d) The available anti-Ra3(NIH) serum was not monospecific. Identification procedures employing CIE and/or CLIE were, therefore, based mainly upon

the position and morphology of precipitates. Using the various anti-Am.e. antibodies, 2 (non-Ra3) antigens were detected in Ra3-1, and 7 (non-Ra3) antigens in Ra3-NIH (Figs. 4, 5). By CLIE experiments, such as those illustrated in Figure 4, Ags 26, 29a, 30, and 31a were found in Ra3-NIH, and Ags 29a and 30a were identified as weak precipitation bands in Ra3-1.

A clearly defined single electrophoretic component could be detected in Ra3-1 by fixing and staining the first-dimension gel. Since it seemed unlikely that this major component had been detected in the aforementioned QIE experiments, we conducted further experiments using anti-Ra3(NIH) (Fig. 5). This serum gave a single strong precipitation band against Ra3-1 corresponding to the electrophoretic component found on the first-dimension gel (Fig. 5B). With Ra3-NIH, anti-Ra3(NIH) gave 2 major and 3 minor bands (not shown). Also, another analysis (not shown) of a mixture

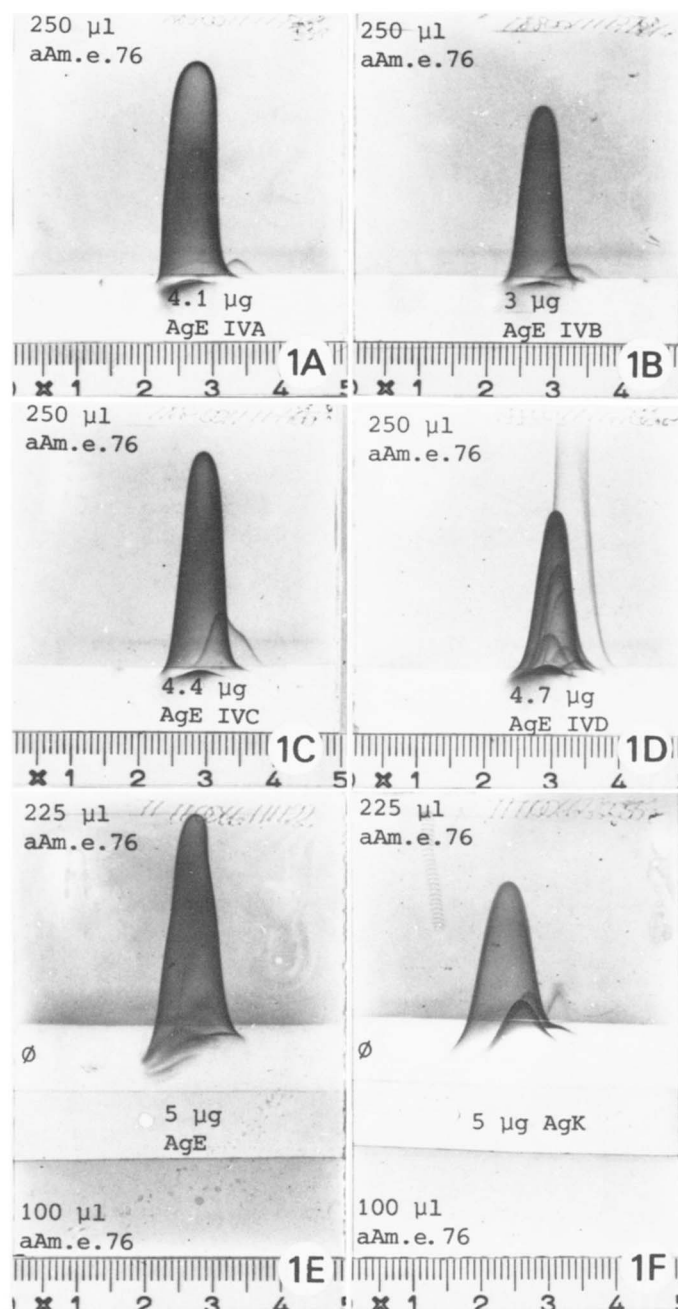


Figure 1. Crossed immunoelectrophoretic analysis of the AgE-IVA, IVB, IVC, IVD, and NIH and of AgK-A against anti-Am.e.76 are shown in A, B, C, D, E, and F, respectively. In this and subsequent figures the anodes are to the right (first dimension) and uppermost (second dimension), according to convention, and the amounts and sources of antigens and antibodies are indicated. See text for further details.

TABLE I

Analysis of purified antigen preparations by crossed immunoelectrophoresis and crossed line immunoelectrophoresis

Antigen Prep.	Major Antigen		Impurities		
	Identification ^a	Content (%) ^b	Number clearly visible	Number faintly visible	Identified Ags ^c
AgE IVA	10E	98	3	2	4, 10K, 11
AgE-IVB	10E	98	2	2	10K, 11, 27a
AgE-IVC	10E	95	2	1	11
AgE-IVD	10E	63	7	0	11
AgE-NIH	10E	90	5	1	10K, 11, 27a
AgK-A	10K	93	3	3	10E, 11, 18, 27a
Ra3-1	N.D. ^c	N.D.	2	0	29a, 30a
Ra3-NIH	N.D.	N.D.	5	2	26, 29a, 30, 31a
Ra5-1	37	N.D.	0	1	N.D.
Ra5-2	37	N.D.	6	0	N.D.
Ra5-3	37	ca. 100	0	0	None
Ra6	35	N.D.	0	2	N.D.
Ra6-X	35	83	0	3	32, 34

^a Antigens which could be identified are numbered according to the CIE reference pattern (Reference 1 and Figure 2B).

^b Estimated by mathematical iteration and QIE (see text).

^c Not determined.

of Ra3-1 and Am.e.80 in an anti-Am.e.80 system, with and without anti-Ra3(NIH) in the intermediate gel, demonstrated: 1) the failure to identify Ra3 in this system, and 2) the identification of trace impurities in Ra3-1 (especially Ag 29a). Analysis of anti-Ra3(NIH) showed marked precipitation of Ag 26 and weaker precipitation of Ags 28, 29, and 29a, as well as Ra3.

Antigen Ra5. There was a common major component (identified as Ag 37) in all preparations of Ra5 (Table I). In Ra5-1, one further antigen was observed by CIE and CLIE using the Am.e.76-anti-Am.e.76 system (Figs. 6A and B). The partially purified preparation, Ra5-2, contained at least 7 antigens, among which 1 was precipitated by anti-Ra5 (Fig. 7). As in the case of Ra3, it was necessary to work with high antigen-antibody concentrations in order to obtain unequivocal identification of the components; this enhanced the sensitivity of detecting impurities. Ra5-3 contained only 1 detectable antigen, which was precipitated by both anti-RA5 and anti-Am.e.78 (Figs. 6C and D).

Antigen Ra6. The Ra6 preparation contained 1 major and 2 trace antigenic materials (Fig. 8), and partially purified fraction Ra6-X contained the same major antigen (Ag 35) and 3 impurities, including Ags 32 and 34 (Table I). Similar numbers of antigenic precipitates were obtained using anti-Am.e.(16-min) in further QIE experiments (not shown).

DISCUSSION

Except for Ra3, we were able to identify all the available isolated ragweed allergens as antigenic components in our CIE reference pattern (1). The purity of the available antigen preparations was estimated by mathematical iteration using QIE (Table I). Because of the extreme sensitivity of the assay system, especially at high antigen and antibody concentrations, impurities could be detected in all fractions except Ra5-2. Subsequent to the experiments described in this paper, it was found that the purity of all the AgE and AgK samples, with the exception of AgE-IVD, could be improved when chromatography was performed using Sephadex G100 as previously described (3, 4).

Use of CIE and CLIE enabled us to confirm the strong antigenic relationship between AgE (10E) and AgK (10K) observed by King *et al.* (4, 14). Furthermore, Ags 11 and 27a, which were impurities in most AgE and AgK preparations, were also found to be related to both AgE and AgK. We were, unfortunately, unable to assess the reported (15) antigenic relationship of Ra4 to the AgE-AgK complex because of the unavailability of an authentic preparation of Ra4.

On gel filtration analysis (1), Ags 10E, 10K, and 11 were found mainly in the m.w. region of about 33,000 daltons, but materials

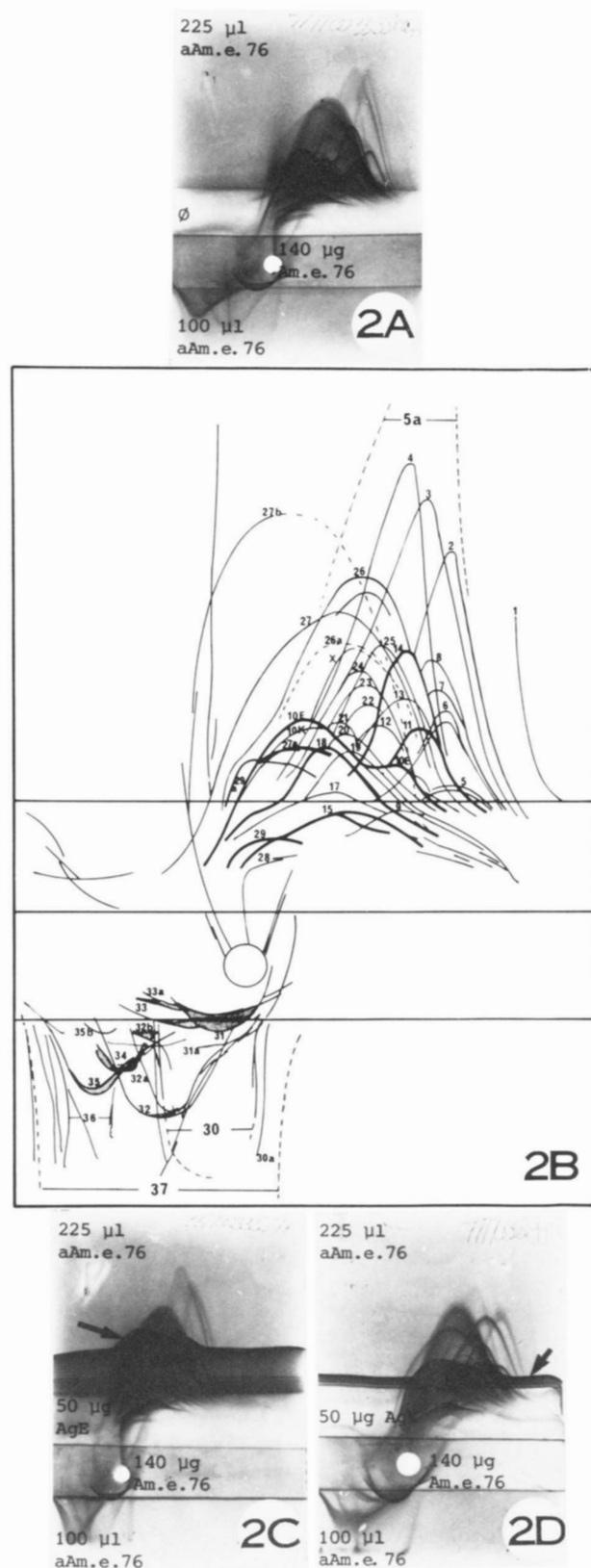


Figure 2. Identification by CLIE of the major antigenic components of AgE-NIH and AgK-A in the standard reference precipitation pattern of Am.e.76. A, CIE reference pattern for Am.e.76 against anti-Am.e.76. B, a drawing of A with classification numbers (from Reference 1). C, as A, except that AgE was applied in the line gel. D, as A, except that AgK was applied in the line gel. When the fused (line-plus-crossed) immunoprecipitates in C and D are compared with the control crossed-immunoprecipitates (A), the major antigenic components of AgE-NIH and AgK-A can be identified as Ags 10E and 10K, respectively (indicated by arrows).

with antigen E- and K-like immunoreactivity were found also in regions of about 18,000 daltons. Antigen E consists of 2 noncovalently bound peptides of about 26,000 and 12,000 daltons (15, 16), which can be dissociated under denaturing conditions. Analysis of the problem of these 18,000-dalton materials and their relation to native α and β chains of AgE has not yet been possible, since previously prepared α and β components were aggregated and/or denatured and, in these forms, are essentially unreactive with AgE-specific antibodies (16).

The pI values of Ags 10E and 10K (pH 5.0 and 5.8) found in our

previous study (1) correspond to the values of pH 5.05 for AgE (mean for components IVB and IVC) and pH 5.9 for AgK-A, respectively (9). Antigen 10E possessed minor isoelectric variants with pI values of 5.8 (similar to the major AgK variant) and 4.4 (similar to Ag11), and Ag 10K possessed a minor variant with a pI of 5.0 (like AgE). The AgE-AgK family therefore appears to be even more complex than previously recognized. The complex interrelationship and variability in m.w. of the members of the AgE-AgK family suggest that conventional immunochemical quantitation of AgE in aqueous extracts of ragweed or derivative fractions may prove to be unreliable.

The Ra3 preparations contained a major electrophoretic component (readily detectable by fixing and staining the first dimension gel) that did not appear in the CIE reference precipitation pattern because of insufficient anti-Ra3 in any of the anti-Am.e. antibody preparations. Using very high antigen-antibody concentrations, 2 trace impurities, Ags 29a and 30a, were detected in Ra3-1. On the

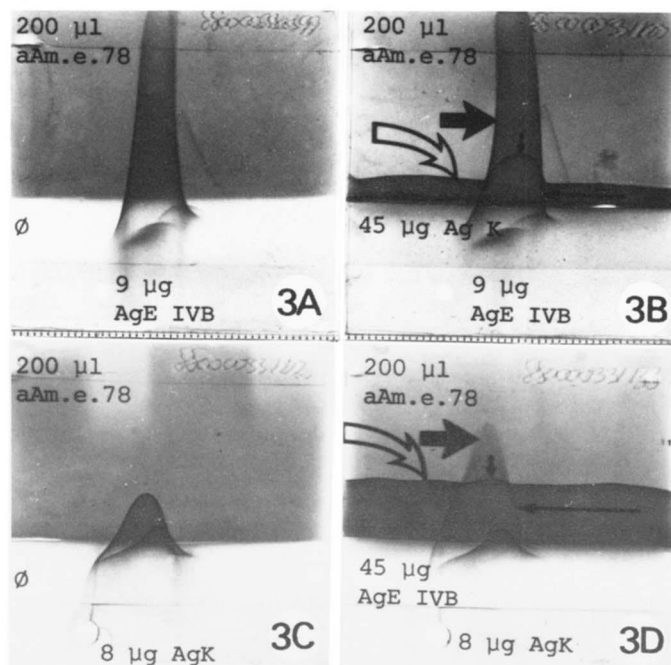


Figure 3. Immunochemical comparison of the major antigenic components of AgE-IVB and AgK-A using CIE and CLIE techniques. A and C, CIE of AgE and AgK, respectively, against anti-Am.e.78. B and D, CLIE with AgE and AgK applied in the wells and AgK and AgE incorporated in the line gels, respectively. Interpretation: In Figures B and D, the large open arrows indicate antigens precipitated by antibodies against AgK and AgE, respectively; the large solid arrows indicate antigens precipitated by antibodies reacting with common determinants on AgE and AgK, respectively; the small arrows indicate antigens precipitated by antibodies more specifically directed against AgK and AgE, respectively; and the long arrows indicate antigens precipitated by antibodies specifically directed against AgE and AgK, respectively.

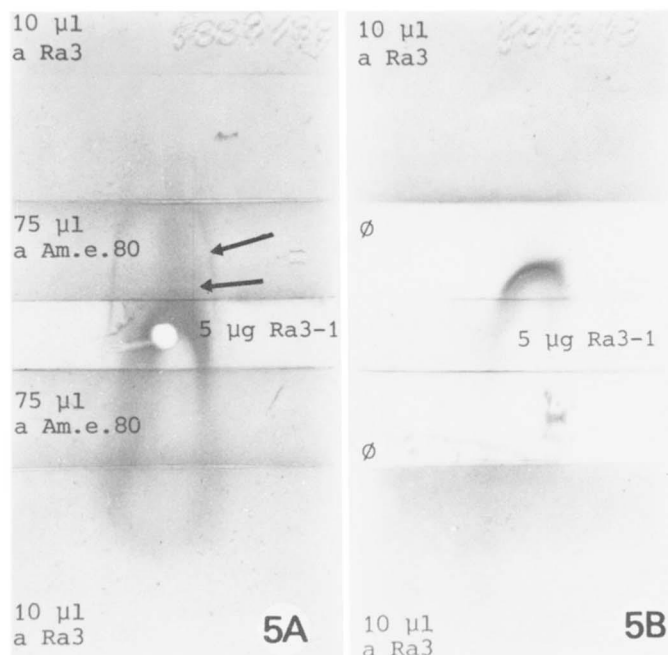


Figure 5. Immunochemical characterization of Ra3. A and B, CIE analyses of Ra3-1 against anti-Ra3(NIH) with and without anti-Am.e.80 added in the intermediate gel. Comparison of A and B shows that addition of anti-Am.e.80 gives rise to 2 further precipitates (arrows in A).

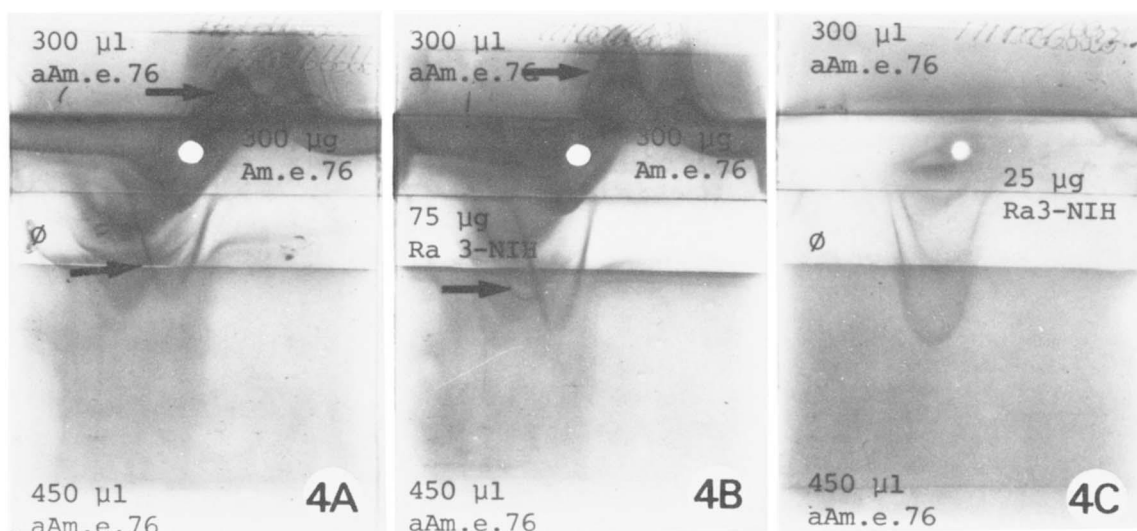


Figure 4. Characterization and identification of some components of Ra3-NIH by means of CIE and CLIE. A, CIE of Am.e.76 against anti-Am.e.76 emphasizing basic components. B, as A, but with Ra3-NIH incorporated into the line gel. C, CIE of Ra3-NIH against anti-Am.e.76. The upper and lower arrows in A and B identify 2 of the antigens of Ra3-NIH (Ags 26 and 30a, respectively).

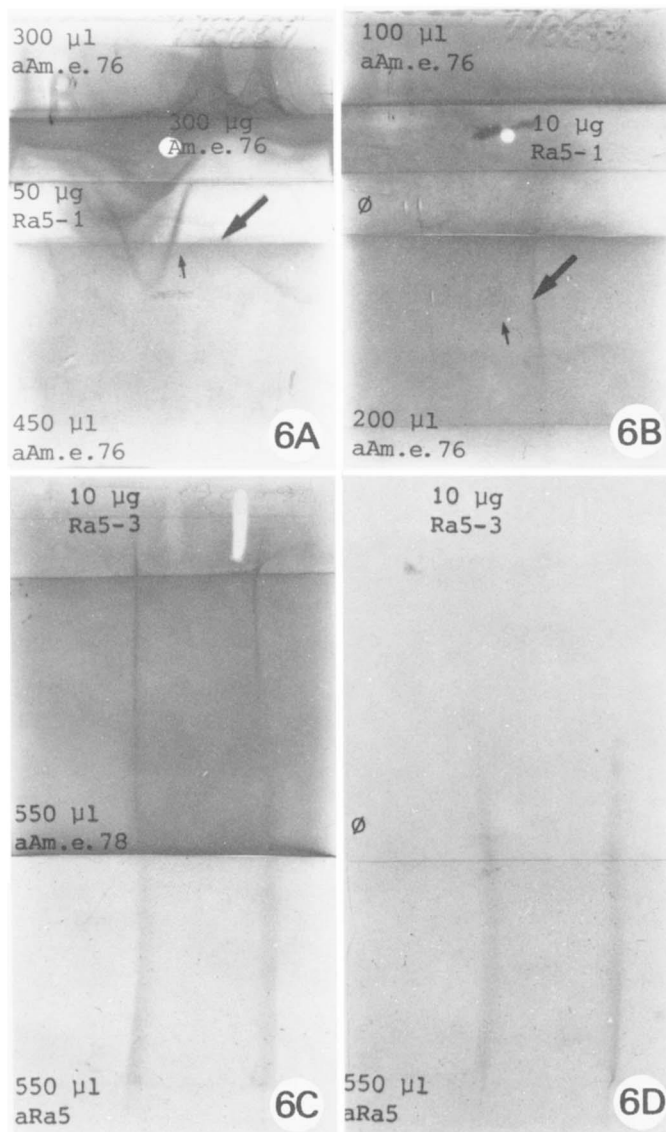


Figure 6. Characterization and identification of Ra5-1 and Ra5-3. A, CLIE of preparation Ra5-1 (as experiment in Fig. 4A except that 50 µg of Ra5-1 was incorporated into the line gel). B, CIE of Ra5-1 against anti-Am.e.76. C and D, characterization and identification of the antigenic component of Ra5-3 using anti-Ra5 in C and a combination of anti-Am.e.78 and anti-Ra5 in D. In A, the 2 fused immunoprecipitates indicated by large and small arrows demonstrate the immunochemical reactions of identity for 2 antigens (Ag 37 and an unidentified antigen) with antigens in the CIE reference pattern. In B, the arrows indicate 2 antigenic components of Ra5-1 (major component, Ag 37, by large arrow and trace impurity by small arrow). The experiments shown in C and D illustrate that Ra5-3 contains Ag 37 with no detectable impurities at the same antigenic load (10 µg) as Ra5-1.

other hand, several impurities were detected in Ra3-NIH, among which the major one, Ag 26, frequently binds patients' IgE antibodies in CRIE⁵. The problem of identifying the impurities in Ra3-NIH was further complicated by the multispecificity of anti-Ra3(NIH) serum, although the major precipitate must be defined as Ra3. Estimation of the amounts of impurities in Ra3-1 and Ra3-NIH was impossible by QIE because of the failure of the anti-Am.e. preparations to precipitate Ra3 effectively at pH 8.6. This was the result of very low (but detectable) anti-Ra3 contents of anti-Am.e. sera and the fact that Ra3 and anti-Ra3 antibodies have pI values at (or around) pH 8.6. However, judged from the density of the precipitate of the unidentified protein and protein-chemical analysis carried

⁵ L. Lowenstein and D. G. Marsh. 1980. Antigens of *Ambrosia elatior* (short ragweed) pollen. III. Crossed radioimmuno-electrophoresis of ragweed-allergic patients' sera with special attention to quantification of IgE responses. In preparation.

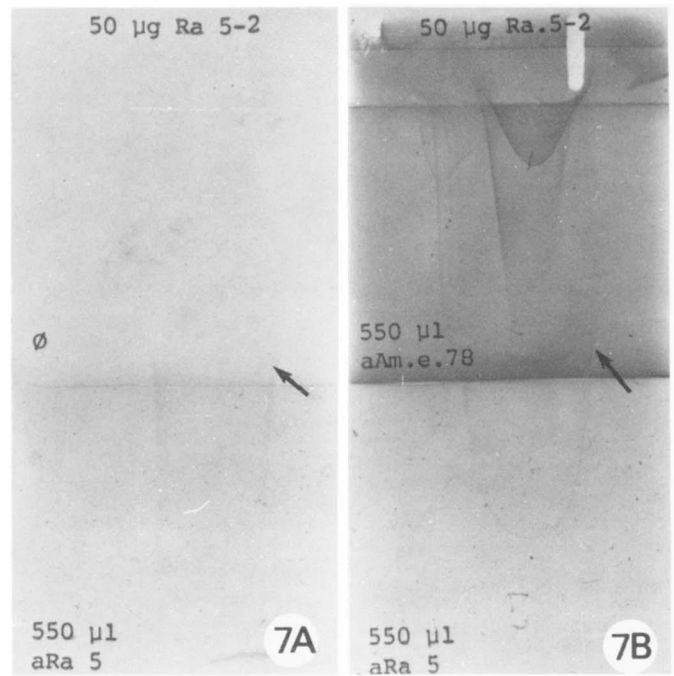


Figure 7. CIE analysis of the partially purified fraction Ra5-2. A, CIE of Ra5-2 against monospecific anti-Ra5. B, as A, but with anti-Am.e.78 incorporated into the intermediate gel. The monospecific antiserum clearly identifies Ra5 labeled by arrows.

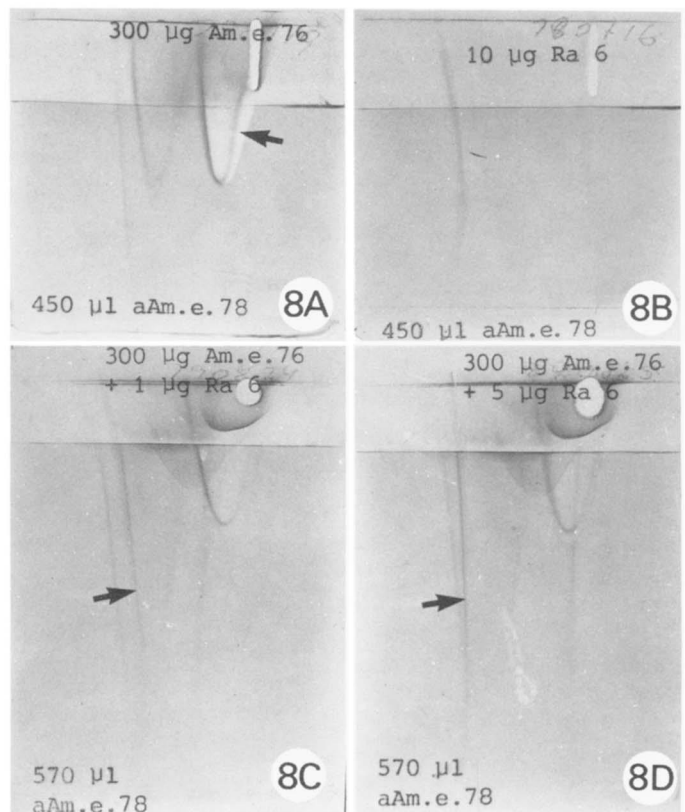


Figure 8. Characterization and identification of Ra6. A and B, CIE analysis of Am.e.76 and Ra6, respectively, using anti-Am.e.78. C and D, identification of the major antigenic component of Ra6 in the CIE reference pattern by means of addition of Ra6 to Am.e.76. In A, an arrow indicates a clear zone in the agarose gel due to a proteolytic enzyme, Ag 30a. B shows 1 major and 1 minor antigenic component in the Ra6 preparation. In D, 5 times more Ra6 was applied than in C. The arrows in C and D indicate Ag 35 which increases significantly by increasing the amount of Ra6 added, thus identifying the major antigen.

out by SDS-PAGE, the purity of Ra3-1 might approach 100%, whereas the purity of Ra3-NIH is certainly less than this.

The identification of Ag 37 as Ra5 (Figs. 6 and 7) was supported by the low m.w., basic pI, high diffusion rate, and weak immunoprecipitate for this antigen. It is likely, however, that the allergenic activities of certain Ra5 preparations, particularly partially purified fractions like Ra5-2, are due in part to antigenic impurities. This conclusion is supported by the finding in a recent study (8) that the frequency of ragweed-allergic patient responsiveness to Ra5-2 (40%) was higher than the value of 17% previously found toward highly purified Ra5 (17).

The major antigen in the Ra6 and Ra6-X preparations was clearly identified as Ag 35, although minor impurities were present in both samples. We have recently demonstrated the importance of this antigen as an allergen in humans by skin testing a large number of allergic patients⁴ and by studying ragweed-allergic patients' sera by CRIE⁵.

Precise quantification of the weight percent of impurities in the isolated allergens was difficult because the allergens themselves are only minor components of the complex allergenic extract and calculation by iteration is prone to errors arising from the preliminary assumptions used.

It is readily apparent from these and previous studies (1, 13) that antigenic identification by QIE technology is critically dependent on the quality of the antiserum used. Anti-Am.e.76 (raised in 3 rabbits) and similar antisera to Am.e.78 and Am.e.80 were all able to detect about 50 ragweed antigens, which is a far greater number than previously recognized using other techniques. In spite of this, the important ragweed allergen Ra3 could not be identified in the CIE reference because of the low content of anti-Ra3 in the anti-Am.e. antibody pools. Obviously, one needs to use further antisera against purified and partially purified fractions (such as Ra3-NIH) and combine CIE with appropriate physicochemical analyses to ensure that occasional problems of antigen identification can be resolved.

This paper also demonstrates the extreme sensitivity of QIE techniques in detecting trace impurities: only 1 preparation, Ra5-3, was found to be immunochemically pure by these methods. However, at the time that most of the known ragweed allergens were isolated, these ultrasensitive QIE methods were not available. We recommend that future analysis of purified allergen preparations include CIE to control for possible impurities, particularly those preparations identified as allergens on CRIE (13). Immunoglobulin E-mediated reactions toward such impurities might other-

wise be interpreted as reactions toward the major component. Such controls are particularly advisable in genetic studies of specific response to purified allergens.

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