

ANTIGENS OF *AMBROSIA ELATIOR* (SHORT RAGWEED) POLLEN

III. Crossed Radioimmuno-electrophoresis of Ragweed-Allergic Patients' Sera with Special Attention to Quantification of IgE Responses¹

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Short ragweed allergenic extract has been studied by means of crossed radioimmuno-electrophoresis (CRIE) with the use of sera from 37 allergic patients and the relevant control sera. In this study 22 of 52 antigens, detectable in crossed immunoelectrophoresis (CIE) against polyspecific rabbit anti-ragweed IgG, were able to bind specific human IgE to their corresponding immunoprecipitates. This binding was semiquantified by comparison with the binding of a standard serum pool. Nine antigens were identified as important allergens, including the previously isolated components, AgE, AgK, and Ra6. Certain allergens (e.g., AgE, AgK, and Ag 31) bound IgE in almost all patients' sera, whereas others showed a bimodal distribution for sera of responder and non-responder patients. The total CRIE score was found to correlate significantly both with ragweed-specific serum IgE antibody determined by RAST ($r_s = 0.88$; $p < 0.001$) and with total IgE level ($r_s = 0.55$; $p < 0.01$). Patient's CRIE scores to AgE also correlated significantly with their specific IgE antibody to AgE measured by RIA ($r = 0.47$; $p < 0.01$) and with skin-test sensitivity to AgE ($r = 0.44$; $p < 0.05$). It was concluded that CRIE is well suited for identification of important ragweed allergens without the previous need for laborious isolation procedures.

Previous studies by Løwenstein and Marsh (1) demonstrated the presence of a complex mixture of at least 52 different antigens (Ag) in short ragweed (*Ambrosia elatior*) pollen extract by crossed immunoelectrophoresis (CIE)² performed with hyperimmune rabbit antiserum toward crude ragweed extracts. Four previously isolated ragweed allergens (AgE, AgK, Ra5, and Ra6) were subsequently identified within the CIE reference pattern (2). The existence of such a large number of ragweed components that are recognized as antigens by rabbit antisera led us to question whether there may be further ragweed allergens that had not previously been identified and isolated.

Previous isolation of pollen allergens was achieved by a combination of physicochemical separation procedures and

analysis of purified fractions by using potency assays such as skin testing, leukocyte histamine release, or radio allergosorbent tests (RAST) (3, 4). In addition, the distributions of allergenic activity in extracts of ragweed and other pollens has been studied by using techniques such as starch and agarose gel electrophoresis followed by cutting out the separated bands and analysis by skin testing (5, 6). Alternatively, a combination of isoelectric focusing and RAST (IEF-RAST) has been applied with a similar aim in view (7). Immunodiffusion and immunoelectrophoresis have been used to identify Ag within partially purified fractions, but it is still not clear which of the components recognized as antigens by animal antisera are important allergens in humans.

The present paper reports the use of a more discriminating technique, crossed radioimmuno-electrophoresis (CRIE), to evaluate the antigenic components of short ragweed pollen in terms of their relative allergenic importance in allergic patients. By a semi-quantitative analysis of the CRIE "allergoprints" for the group of patients studied, we have been able to identify further allergenic components of ragweed.

MATERIALS AND METHODS

Patients. The 37 untreated patients in this study were a subset of people participating in various clinical and immunologic studies of ragweed allergy at The Good Samaritan Hospital. The general characteristics of these patients have been described in detail elsewhere (8). In brief, the group consisted of Caucasians (19 male, 18 female) having a mean age of 30 ± 10 yr SD. All were highly sensitive to *Ambrosia elatior* (short ragweed) pollen by clinical criteria and by skin testing with the crude pollen extract. We also included several Danish control patients who were highly sensitive to pollens, animal danders, housedust mites, and fungi in various combinations. They were not, however, sensitive to ragweed pollen, because this is not found in Denmark. A pool of sera from 1000 nonallergic Danish individuals and the serum from an IgE myeloma patient (PS) were used as additional controls.

In order to perform the semi-quantitative CRIE analysis, a "standard serum" pool was prepared from equal quantities of the sera from six of our patients, who were found to have high IgE antibody levels toward 20 of the detectable Ag within the ragweed system (all of the Ag toward which we were able to detect IgE antibody in this study except Ag 21 and Ag 34).

Crossed radioimmuno-electrophoresis (CRIE). The general principles of the CRIE technique are discussed in detail elsewhere (9). CIE was performed as described (1), by using dialyzed crude ragweed extract (Am.e.76) and a pooled IgG fraction of anti-Am.e.76 from three rabbits. Unlike the normal CIE experiments, the plates were dried in cold air and were not stained with Coomassie Blue. CRIE was performed on the CIE immunoplates at 18 to 24°C as follows: 1) Seven milliliters of 1/15 M phosphate buffer (pH 7.5) and 0.7 ml of each patient's serum were added to plastic boxes containing the plates (the boxes were slightly larger than the plates, and the amount of serum was just sufficient to cover the gel film on each plate); 2) after allowing the serum IgE antibodies to react with free antigenic determinants in the precipitates on the immunoplates, the unbound serum proteins were removed by washing with 10 to 20 ml of 0.1 M sodium chloride at least four times for 10-min periods; 3) 0.35 μ Ci (approximately 200,000 cpm) of ϵ -chain-specific polyclonal ¹²⁵I-anti-IgE (10) in 7 ml of incubation buffer (0.05 M phosphate buffer, pH 7.5, containing 0.3% [w/v] of bovine serum albumin, 0.9% [w/v] of EDTA²) was added and allowed to react for 24 hr;

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² Abbreviations used in this paper: Am.e.76, 1976 collection of the pollen of *Ambrosia elatior* (short ragweed); CRIE, crossed radioimmuno-electrophoresis; IEF, isoelectric focusing; RAST, radioallergosorbent test; RIA, radioimmunoassay; CIE, crossed immunoelectrophoresis; EDTA, ethylenediaminetetraacetic acid; AU, arbitrary unit; AgE, antigen 10E; AgK, antigen 10K.

4) after four 10-min washings with 10 to 20 ml saline and one washing with distilled water, the plates were dried in hot air; 5) autoradiography of the plates was performed sequentially during days 0 to 3, 3 to 7, and 7 to 20 (corresponding approximately to incubation periods of 3, 4, and 12 days when corrected for radioactive decay), and the resultant x-ray films were developed and evaluated; 6) finally, the plates were stained with Coomassie Brilliant Blue (1).

Grading system. Ag-specific IgE responses toward each ragweed Ag were evaluated in arbitrary units (AU) by visual comparison of the radiostaining produced by CRIE analysis of each patient's serum relative to that produced by similar analysis of the standard serum. Table I shows the grading system, together with an example of how the system operates. According to the results of CRIE performed with the negative control sera, measurable nonspecific binding of radioactive anti-IgE occurred only with Ag 10E (AgE) and Ag 10K (AgK) (see Fig. 1 for the numbered reference pattern). It was necessary to reduce the AU values for antibodies toward each of these Ag by a value of 1. In the present studies, there was no significant nonspecific coprecipitation (9, 11).

Further control experiments were performed by mixing the standard serum pool with increasing amounts of IgE myeloma serum (PS). No increase in radiostaining was observed when mixing serum PS and our serum pool in proportions of 1:256 to 1:1.

Skin testing with AgE. Highly purified short ragweed AgE was prepared as previously described (8). Thirty-four patients were skin tested by the quantitative intradermal skin-test technique, with the use of concentrations of AgE between 10^{-7} and 1 μ g/ml. A patient's sensitivity to AgE was expressed as that concentration that induced a 2+ (8- to 10-mm) diameter wheal with erythema response in the skin of the forearm (8).

Radioimmunoassays (RIA). Total serum IgE levels were determined according to the double-antibody inhibition procedure of Ishizaka *et al.* (12), as described (8). Specific IgE antibodies against dialyzed crude ragweed pollen extract were measured by a RAST assay by using dialyzed short ragweed extract coupled to Sepharose 4B beads (13, 14). Absolute levels of ragweed-specific IgE antibodies were determined in a standard reference serum (13, 15), which was included in the assays. This allowed us to estimate the concentration of ragweed-specific antibodies in nanograms per milliliter.

Specific IgE antibodies to AgE were determined by double-antibody radioimmunoassay (RIA), as described elsewhere (16, 17). Only 36 out of 37 sera were available for this study. In brief, ^{125} I-labeled AgE was incubated with the patient's serum (diluted 1/2 to 1/10 depending on the IgE antibody titer) for 4 hr at 23°C. AgE-IgE antibody complexes were allowed to precipitate by incubation overnight at 4°C after the addition of goat anti-IgE (Fc-specific) plus IgE (PS) myeloma carrier. The precipitates were washed and counted. After subtraction of "blank" values, the levels of specific IgE antibody to AgE were determined by comparison with a reference curve of a standard serum pool of known antibody content.

RESULTS

The CIE reference pattern is illustrated in Figure 1. Figure 2 shows the sequential radiostaining at 1, 2, 3, 5, 7, and 10 days by using the standard serum pool. After 1 day, radiostaining of the AgE-AgK complex (AgE, AgK, and Ag 11) is apparent; by 3 days, Ag 26 and Ag X are faintly visible; after 5 days, some cathodically migrating Ag are visible; and after 10 days the complete CRIE pattern is apparent.

Figure 3 shows representative allergoprints with sera used from six allergic patients and a negative control. The bottom right-hand picture shows the CIE reference precipitation pattern stained with Coomassie Blue. Immunoglobulin E antibodies of all of these patients bind to AgE. The control plate, comprising the pool of Danish allergic sera, shows only a faint radiostaining corresponding to AgE and AgK, which bind ^{125} I-anti-IgE nonspecifically to a small degree (1 AU). Similar data were obtained by using other control sera from nonallergic American patients. The six allergoprints differ both qualitatively and quantitatively in their binding of IgE to the various ragweed antigens present in the CIE immunoprecipitates. The sera that exhibit a high degree of binding to AgE also show IgE binding to many other antigens.

As previously indicated, the radiostained precipitates were quantified as shown in Table I. In order to evaluate the reproducibility of our scoring system, the same CRIE autoradiographs of six patients were reevaluated independently several

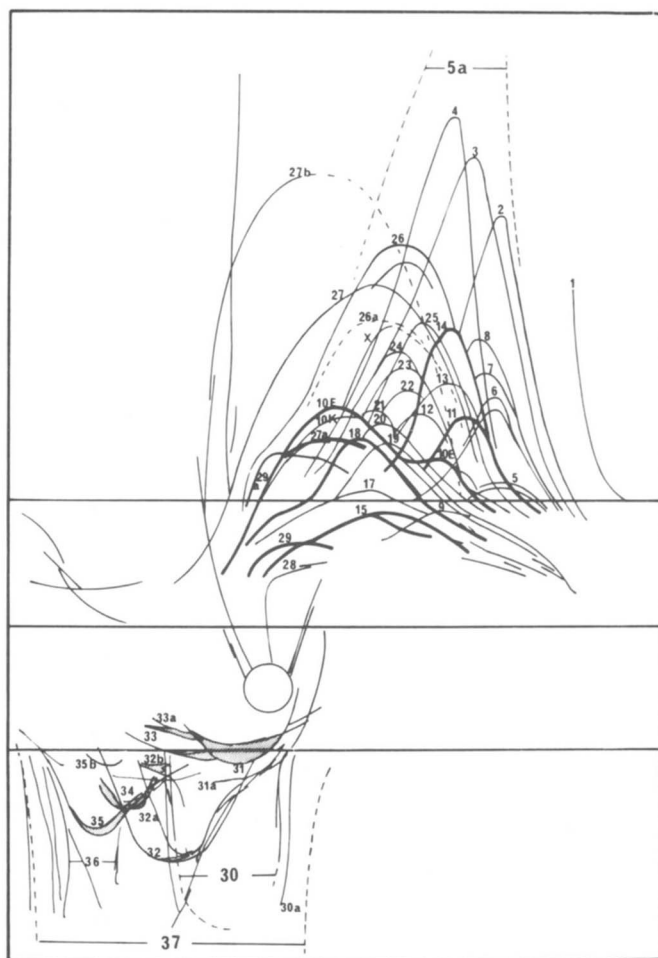


Figure 1. Numbered CIE reference pattern for the ragweed-anti-ragweed system used in these studies (1).

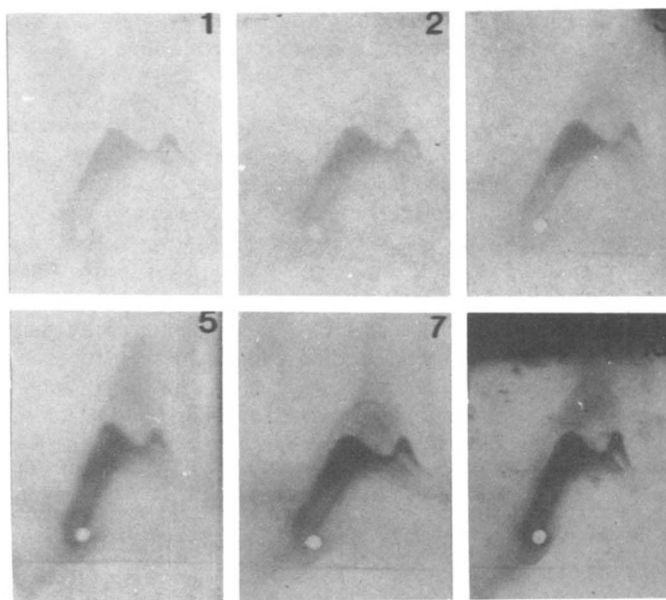


Figure 2. Analysis of the standard serum pool by CRIE. Autoradiography was performed for increasing time periods (days) as indicated on the individual x-ray films.

months after the first evaluation. We found an excellent correlation between the CRIE scores for all allergens in all of these patients ($r_s = 0.99$; $p < 0.001$). In order to evaluate the reproducibility between different CRIE plates, we performed

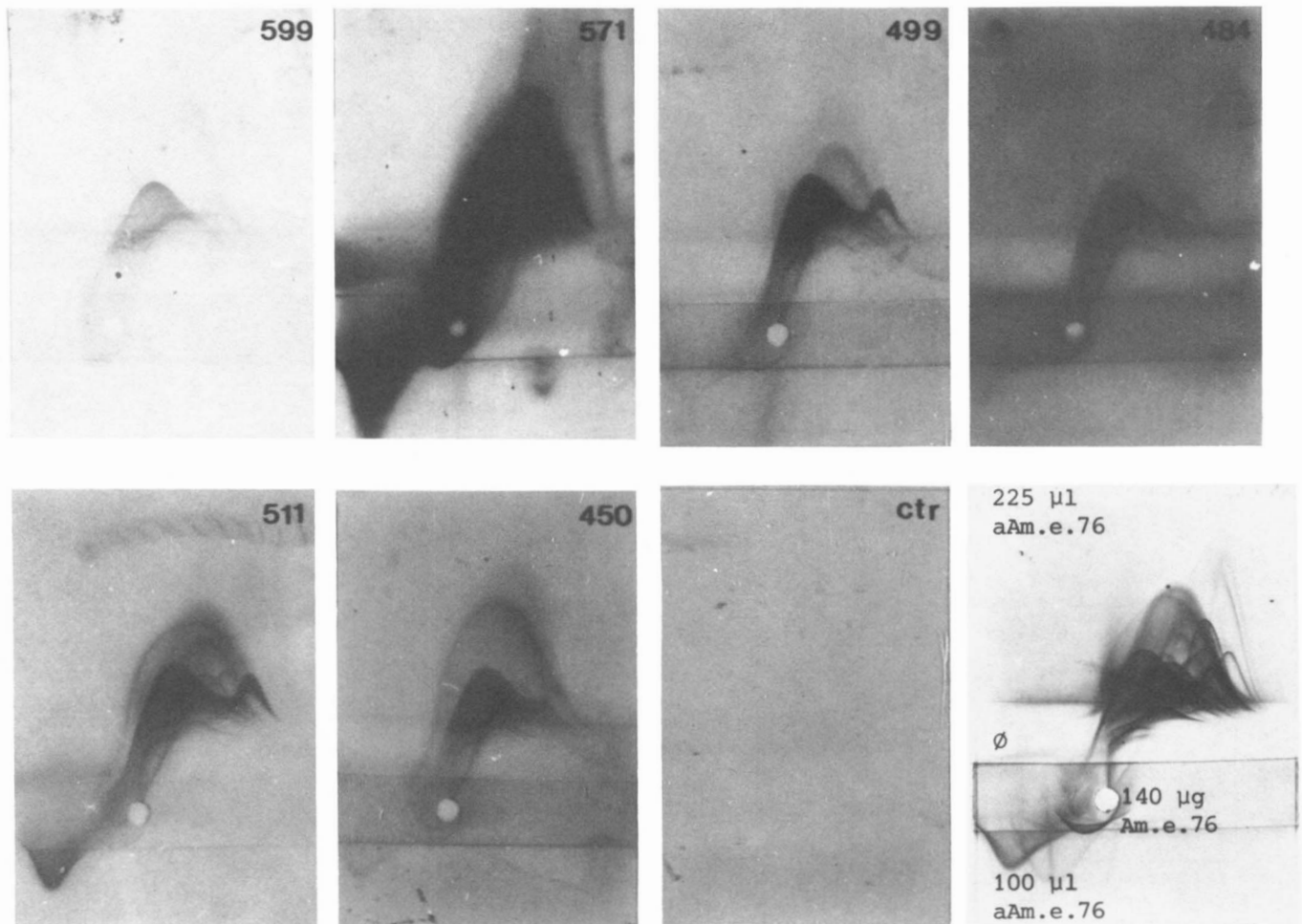


Figure 3. CRIE performed by using the sera of six different ragweed-allergic patients (identification indicated) and a control serum (ctr) from a pool of 1000 normal individuals. The autoradiography was carried out for 12 days. The Coomassie Blue-stained CIE reference pattern is included at the bottom right of the figure.

TABLE I
Grading system for CRIE analyses of patients' IgE antibody responses toward individual ragweed antigens^a

Autoradiography of the Patient's Serum (days)	Autoradiography of Standard Serum Pool (days)					
	1	2	3	5	7	10
	SCORE (AU)					
12	1	2	3	5	7	10
4	3	6	9	15	21	30
3	4	8	12	20	28	40

^a Use of this table: A score of 15 AU for a particular patient's IgE binding to a specific immunoprecipitate corresponds to the case where the intensity of radiostaining after 4 days of autoradiography of the patient's serum is equal to that obtained after 5 days of autoradiography for the standard serum pool.

two independent experiments with five patients' sera and one nonallergic control serum. Again, our results showed excellent concordance when evaluated by the same person, who did not know the code ($r_s = 0.90$; $p < 0.01$).

Figure 4 shows the distribution of CRIE scores for each of the 22 Ag toward which we observed IgE binding. Certain Ag, e.g., AgE, AgK, and Ag 31, exhibited IgE binding with almost all patients' sera. Other Ag (e.g., Ag 5A, Ag X, Ag 26, and Ag 27) showed a bimodal distribution of IgE binding in that there were well-defined, relatively large groups of both responder and nonresponder individuals. Only a few exceptional allergic individuals responded to Ag 37 (Ra5) and to Ag 21 and Ag 34.

In order to obtain an estimate of the precision of our CRIE analysis in relation to IgE antibody levels toward crude ragweed extract, we analyzed IgE antibody by RAST vs the total CRIE

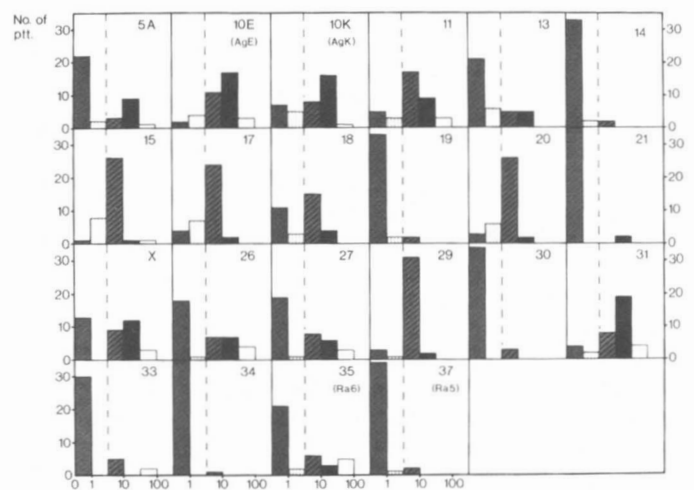


Figure 4. Distribution frequency of IgE antibody responses to individual allergens obtained by CRIE. Allergens are numbered according to our CIE reference pattern (Fig. 1), and the identification of previously isolated components is indicated. CRIE scores (■, 0 to 1; ▨, 2 to 3; ▩, 4 to 10; ■, 11 to 30; □, ≥ 31) to individual allergens were obtained by analyzing the sera of 37 ragweed-allergic patients. The dotted lines indicate the limit between nonsignificant and significant IgE binding. For determination of CRIE scores, see text and Table I.

score. As shown in Figure 5A, there was an excellent agreement between these two parameters by the Spearman rank order test ($r_s = 0.88$; $p < 0.001$). There were also modest correlations between total IgE levels and total CRIE scores

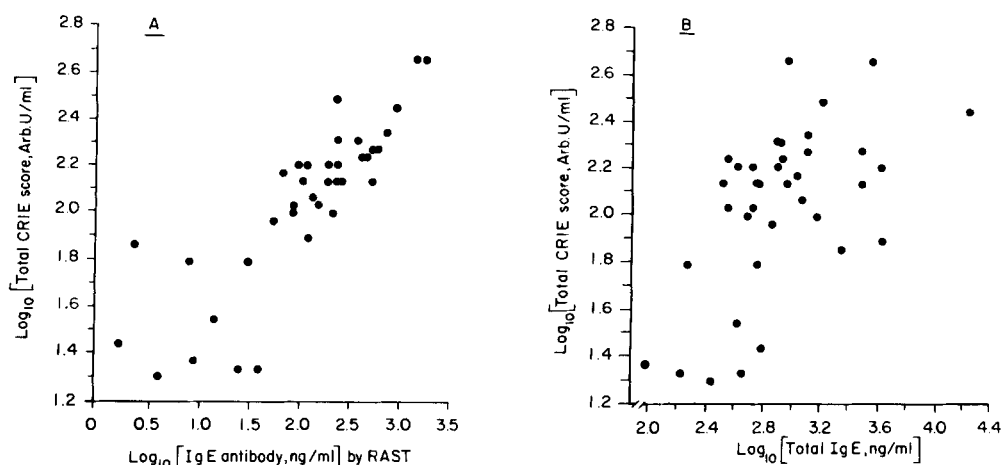


Figure 5. A, comparison of $\log_{10}$ [IgE antibody] response to short ragweed as determined by the RAST vs $\log_{10}$ total CRIE score toward all of the ragweed allergens. B, comparison of $\log_{10}$ [total IgE] vs $\log_{10}$ total CRIE score. Thirty-seven sera were assayed in both experiments.

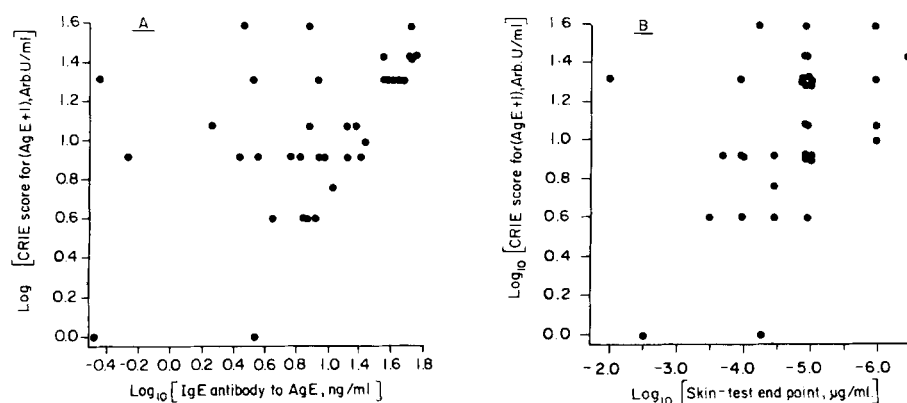


Figure 6. A, comparison of $\log_{10}$ [IgE antibody to AgE] by RIA vs $\log_{10}$ [CRIE score for (AgE + 1)] for 36 sera. B, comparison of $\log_{10}$ [skin-test response to AgE] vs $\log_{10}$ [CRIE score for (AgE + 1)] for 34 individuals.

($r_s = 0.55$; $p < 0.01$), as shown in Figure 5B.

In order to examine the precision of the CRIE analysis with respect to IgE antibody levels toward a single ragweed allergen, we investigated the relationship between the CRIE score to AgE (Ag 10E) and two measurements of IgE response to AgE, namely, RIA and intradermal skin testing (Fig. 6). Before analysis, all data were converted to $\log_{10}$ values, except for CRIE scores where $\log_{10}$ [CRIE + 1] was used, because one individual had a negative CRIE response to AgE. Both associations were significant by linear regression analysis, although not very striking; $r_s = 0.47$, $p < 0.01$ in the case of the correlation with IgE antibody by RIA and $r_s = 0.44$, $p < 0.05$ for the correlation with skin-test response.

Although our data for IgE response to AgE correlated significantly with the CRIE score for this antigen, the same was not true for allergen Ra5. Seven of the allergic patients studied were identified as responders to Ra5 by IgE-antibody determinations and by skin testing (17), but only two of the more sensitive individuals were found to be responders in the CRIE system.

DISCUSSION

Using CRIE analysis, we were able to identify 22 short ragweed Ag that bind human IgE antibodies. These components may therefore be considered to be ragweed allergens. Thus, these experiments reveal a high degree of allergenic complexity of short ragweed pollen extract, which should be considered in evaluating extracts selected for use in immunotherapy, as well as in the evaluation of IgG antibody responses in patients undergoing therapy. It is also quite possible that

several of the remaining 30 Ag detectable in our CIE reference are also allergens in small subgroups of the allergic population, which are not represented in our sample of 37 individuals. The clear advantage of CRIE analysis is that we are able to identify which antigenic components are allergens without the need to isolate each one before assessing allergenic reactivity.

In order to facilitate better quantitation of patients' IgE antibody levels, we developed a new CRIE scoring system relating the patient's IgE antibody level to that found toward the same allergen in a standard serum pool. As seen in Figure 4, it was then possible to make a semiquantitative evaluation of the relative importance of individual ragweed Ag as allergens in allergic humans. In particular, this CRIE system enabled us to identify nine Ag as important allergens, namely, AgE, AgK, and Ag 35 (Ra6), which have been reported previously (18–20),³ as well as Ag 5A, Ag 11, Ag X, Ag 26, Ag 27, and Ag 31, which have not previously been reported. Thus, two-thirds of the important ragweed allergens, which had not previously been revealed by conventional approaches, were clearly defined by CRIE. However, as noted in a previous paper (2), we were not able to detect Ra3 in our CIE reference system, and there might even be other nondetected allergens included in our ragweed system. Those allergens toward which the highest average CRIE score was found among the patients studied were AgE, AgK, and Ag 31; according to a recent classification system (9, 21) they would be designated "major allergens."

The sum of the CRIE scores to the individual ragweed allergens shows an excellent overall correlation with specific IgE to

³ Roebber, M., R. Hussain, D. G. Klapper, and D. G. Marsh. Isolation and properties of a new short ragweed allergen, Ra6. Manuscript in preparation.

crude ragweed determined by RAST assay ($r_s = 0.88$; $p < 0.001$). This can only partly be accounted for by the combined contributions of AgE, AgK, and Ag 11, which represent 24% of the sum of the CRIE scores. There is a weaker, although significant, correlation between the sum of the CRIE scores and total serum IgE levels ($r_s = 0.55$; $p < 0.01$). Because RAST and total IgE values were also correlated ($r_s = 0.51$; $p < 0.01$), this could largely explain the latter correlation. We also found significant correlations between the CRIE score for AgE and a) specific IgE antibody levels to AgE determined by RIA ($r = 0.47$; $p < 0.01$) and b) skin-test sensitivity toward this allergen ($r = 0.44$; $p < 0.05$). The relatively weak correlations in the case of IgE antibody levels to AgE is somewhat surprising because both the RIA and the CRIE techniques should be directly measuring levels of specific IgE in the serum. Possible explanations are that certain important allergenic determinants on the AgE molecule are "blocked" by rabbit antibody in the immunoprecipitates used to bind human IgE in the CRIE technique, or that labeling AgE with ^{125}I masks some of its epitopes.

Although we could easily detect IgE antibody to AgE by the CRIE system, we had difficulty in identifying patients known to be sensitive to allergen Ra5. The principal reasons for this are the very low concentrations of Ra5 Ag in the dialyzed ragweed pollen extract (2.5 $\mu\text{g}/\text{ml}$; Reference 1) and of Ra5-specific IgG antibody in the rabbit IgG pool (2). In fact, the Ra5-anti-Ra5 immunoprecipitate could only be detected at high antigen-antibody concentrations by conventional Coomassie Blue staining of CIE plates (2). At the lower concentrations used in the present experiments, no protein staining was evident. The two patients in whom IgE antibodies to Ra5 were detected were independently found to have very high levels of specific IgE to Ra5 (17). The remaining five Ra5-allergic subjects, in whom no IgE antibody to Ra5 was detected by CRIE, had lower levels of Ra5-specific IgE (17). This rather extreme example of the low m.w. Ra5 allergen (5000) illustrates a potential drawback in CRIE analysis; it relies on an adequate amount of immunoprecipitate for each antigenic component being present in the CIE plate.

In a series of control experiments, we did not observe any nonspecific binding of patients having high IgE antibody levels toward ragweed allergens, with the exception of very weak binding to AgE and AgK, which necessitated a deduction of 1 AU from the CRIE scores. Also, addition of myeloma serum PS to the standard serum pool led to no difference in the resultant CRIE scores. This indicates that nonspecific IgE does not competitively inhibit the binding of specific IgE to the antigen in the CIE immunoprecipitates. We also found that the precision of our scoring system was very high when the radioautographs were evaluated a second time by the same reader. Furthermore, the reproducibility between different experiments performed by using the same serum showed excellent agreement.

The advantage of the CRIE system for allergenic analysis over other systems, such as IEF-RAST (7), are obvious. In our system, we show clear-cut IgE antibody responses toward individual antigens, whereas in the IEF-RAST analyses (7) it is not possible to identify which antigens in the system are actually behaving as allergens. Also, one is relying on the presumption that each allergen has a unique isoelectric point; this is clearly not the case, as shown in our previous experiments (1) and in numerous previous examples of multiple "isoallergenic" forms of allergens (3). A further advantage of the CRIE system is that it allows identification of important new allergens, which can then be isolated by standard physico-chemical procedures. Finally, this system is particularly useful

for genetic studies in that it allows rapid semiquantitative evaluation of patients' IgE responses toward a large array of different allergens without the need to isolate each component. For this reason, we have employed this system for genetic analysis of ragweed-allergic patients' IgE responses in the next paper in this series.⁴

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