

Studies on the immunological relationship of ragweed pollen antigens E and Ra.3

Lawrence Lichtenstein, M.D., Ph.D.,* Marianne Roebber, M.Sc., and Lawrence Goodfriend, Ph.D.

Baltimore, Md., and Montreal, Quebec, Canada

This study compares the allergenic and antigenic activities of ragweed pollen antigens Ra.3 and E using the human leukocyte histamine release assay. Leukocytes from 23 antigen E-sensitive patients were challenged separately with varying concentrations of Ra.3 and E. Six were unresponsive to Ra.3, although the same cells released 60 to 100 per cent histamine on challenge with antigen E. The leukocytes of 16 patients gave maximal release of 50 to 100 per cent with Ra.3. Leukocyte sensitivity to Ra.3 relative to E ranged from 0.1 to 10⁴ and demonstrated little or no allergenic cross-reaction between the 2 antigens. Skin tests on an additional 19 patients confirmed this pattern. Leukocytes were challenged separately with mixtures of each allergen and dilutions of human antisera to antigen E (a-AgE) and water-soluble ragweed (a-AgWSR). Whereas a-AgWSR and a-AgE were equally inhibitory to E-induced histamine release, only AgWSR effectively inhibited Ra.3-induced release. Rabbit antiserum to Ra.3 and AgE in most patients inhibited the homologous allergen and demonstrated little or no cross-reactivity. In 2 patients, however, anti-AgE inhibited Ra.3-mediated histamine release while the homologous antiserum was inactive. We conclude that antigens Ra.3 and E are antigenically and allergenically distinct.

There is a continuing need to identify and purify the allergens of the common pollens that cause clinical allergic symptoms. Efforts in this direction have been carried out over the last several years with ragweed, rye grass, and birch pollens; it appears that the major allergen(s) in each case has been identified.¹⁻⁵ In all three it is a simple protein with no prosthetic groups and a molecular weight at between 20,000 and 40,000. King and Norman¹ have demonstrated that antigen E, of ragweed, is the major allergen of that pollen; in population studies virtually all patients sensitive by skin test to crude ragweed pollen react to antigen E. In a recent study using leukocyte/basophil histamine release, we have shown that about 95 per cent of donors whose cells release histamine on challenge with ragweed also respond to antigen E; the exceptions occur only in individuals who are highly insensitive (by skin test, histamine release, and the degree of clinical illness) to the crude pollen.⁶ Antigen K has also been

From The Johns Hopkins University School of Medicine at The Good Samaritan Hospital, and the Division of Immunochemistry and Allergy, Royal Victoria Hospital.

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Reprint requests to: Dr. Lawrence M. Lichtenstein, The Johns Hopkins University School of Medicine at The Good Samaritan Hospital, 5601 Loch Raven Blvd., Baltimore, Md. 21239.

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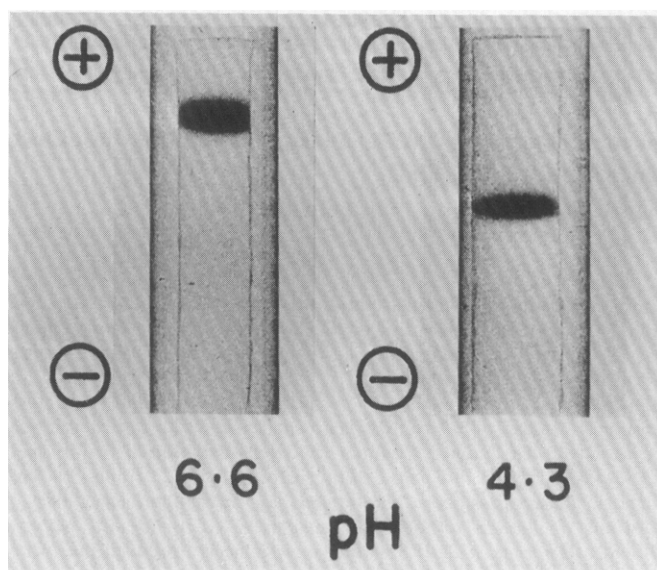


FIG. 1. Polyacrylamide gel disc electrophoresis of antigen Ra.3. Sample load, 15 μ g; running time, 3 hours at 2 mA per tube.

isolated by King, Norman, and Lichtenstein⁷ and shown to cross-react extensively with antigen E. Virtually all individuals reactive to E are reactive to K, and there is a relatively constant ratio of sensitivity to these antigens with E being about twice as active as K.⁷

For many purposes it is useful to identify and purify other ragweed pollen allergens, particularly those that do not cross-react with antigen E and to which the ragweed pollen-sensitive population shows more of a variety in their reactivity. This report further describes antigen Ra.3,⁸ a smaller protein with a molecular weight of 11,000,⁹ which appears to have little or no allergenic or antigenic cross-reactivity with antigen E and to which some allergic individuals are highly reactive while others are completely insensitive.

MATERIALS AND METHODS

Preparation of antigens

Antigen E was provided by Dr. T. P. King of Rockefeller University or by the National Institutes of Health Committee on Allergen Standardization prepared on a contract to Abbott Laboratories.

Antigen Ra.3 was prepared from an aqueous extract of short ragweed pollen (Sharp and Sharp Inc., Everett, Washington) by the method of Underdown and Goodfriend⁸ with modifications.⁹ The isolated product gave a single band on acrylamide gel disc electrophoresis at pH 4.3 and 6.6 (Fig. 1). A single precipitin arc was observed on immunodiffusion in agar with homologous rabbit antiserum (Fig. 2, *A*); however, no precipitin reaction was obtained with rabbit antiserum to antigen E (Fig. 2, *B*). A single precipitin arc was obtained on immunodiffusion test of antigen Ra.3 with rabbit antiserum to whole aqueous ragweed pollen extract.^{8, 9}

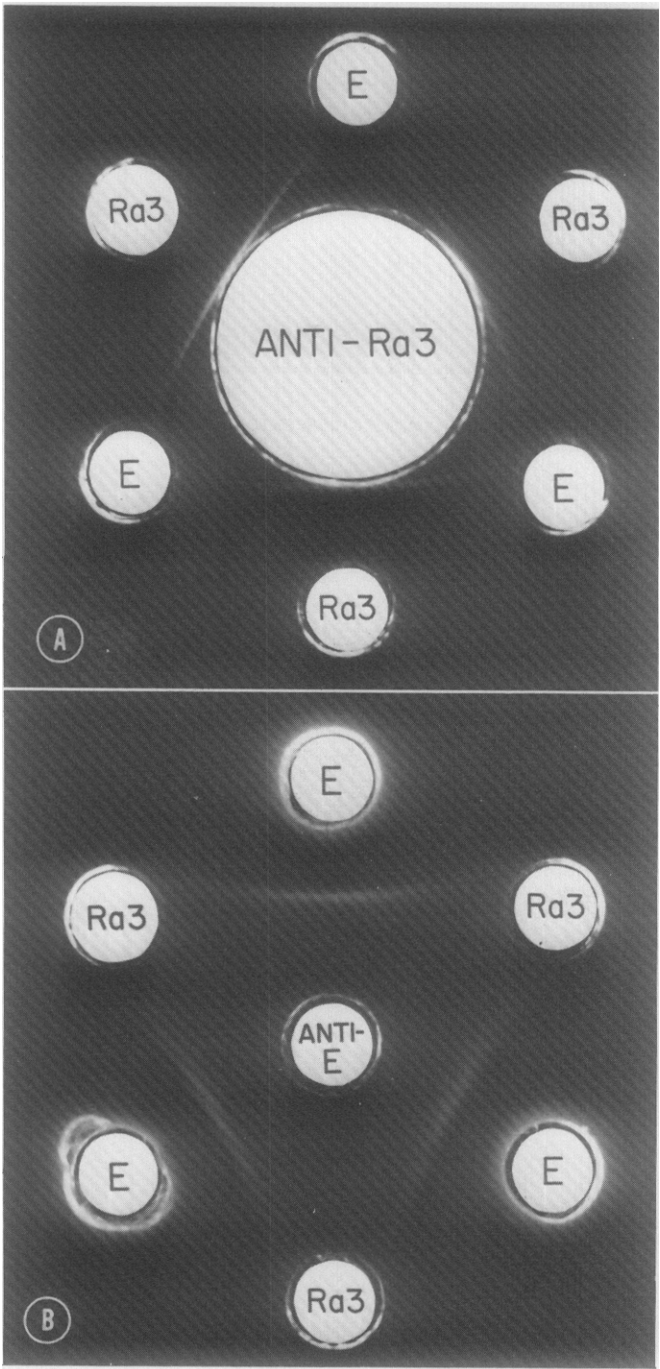


FIG. 2. A and B, Immunodiffusion analysis of antigens Ra.3 and E with corresponding antisera. The antigens were examined at concentrations of 0.2 mg. per milliliter, respectively.

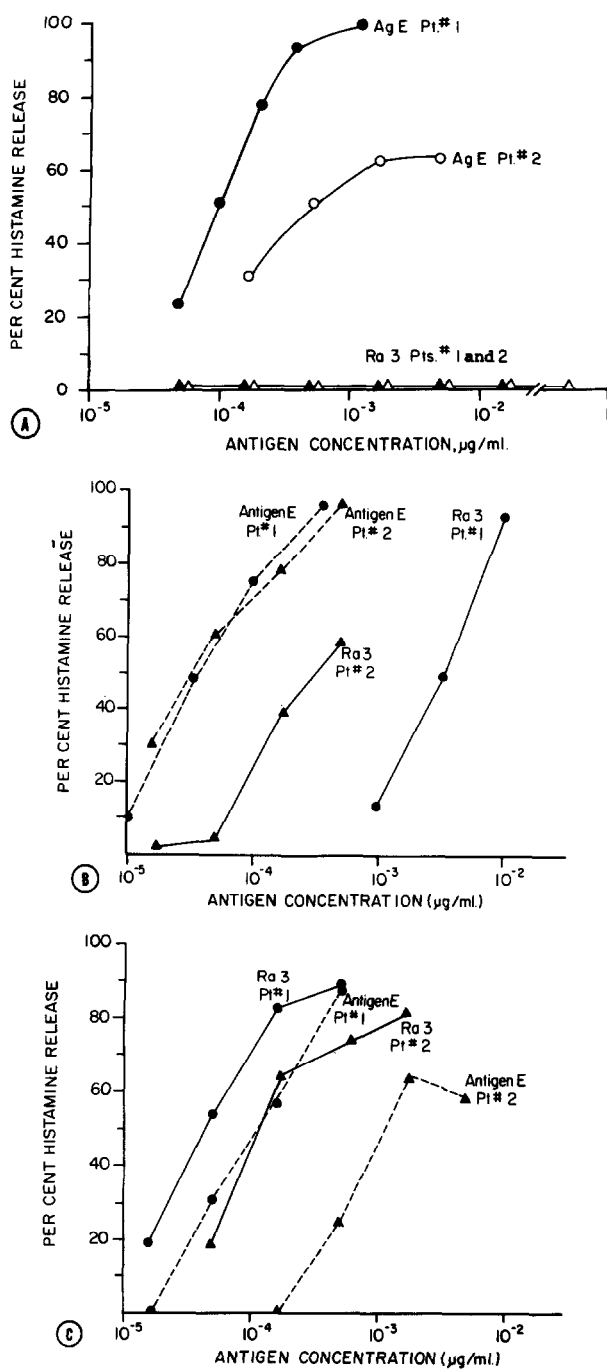


FIG. 3. Patterns of histamine release on challenge of the leukocytes of different patients with ragweed antigens E and Ra.3. **A**, Two patients sensitive to antigen E but totally unreactive to antigen Ra.3. **B**, Two patients sensitive to both antigens but more highly sensitive to antigen E. This is the usual pattern. **C**, Two patients more sensitive to antigen Ra.3 than to antigen E.

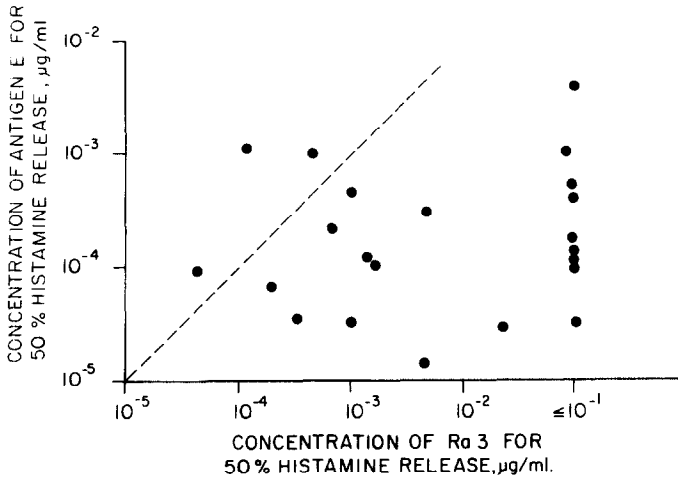


FIG. 4. Plot of all histamine release data. Each point is the sensitivity of one patient to both antigens. The dashed line represents equal sensitivity to both antigens.

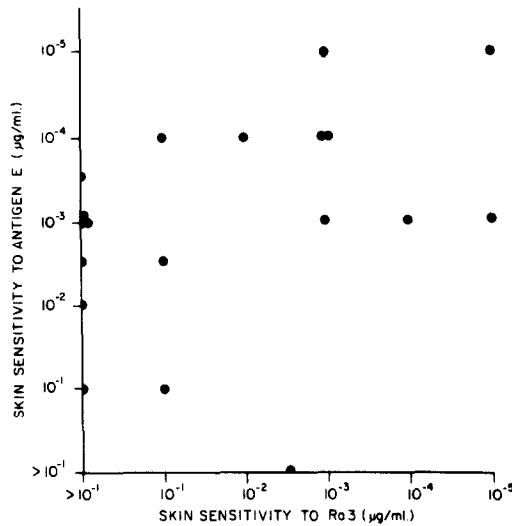


FIG. 5. Skin sensitivity to ragweed antigens E and Ra.3. Each point is the sensitivity of one patient to both antigens. The skin test titers represent an estimate of a midpoint (2+) sensitivity range.

Preparation of antisera

The rabbit anti-E was made by 3 weekly injections of antigen E in Freund's incomplete adjuvant with the animal being bled 10 days after the last injection. The total dose of antigen E administered was 1.6 mg.

The human anti-whole ragweed pollen extract and anti-antigen E was a pool from the serum of 10 donors, each of whom had been treated with the appropriate antigen for ragweed hay fever. The doses of antigen were variable.

Rabbit anti-Ra.3 was raised by 4 weekly injections of antigens Ra.3 in Freund's incomplete adjuvant with the animal being bled 7 days after the last injection. The total dose of antigen Ra.3 was 4 mg.

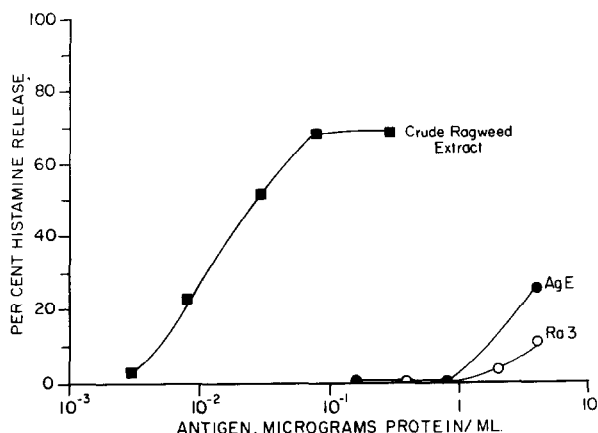


FIG. 6. Histamine release from the cells of a patient reactive to whole ragweed pollen extract at a high concentration but insensitive to antigens E. and Ra.3.

Histamine release

The techniques have been described previously and represent the May micromodification of the procedure described by Lichtenstein and Osler¹⁰ and May and associates.¹¹ Blocking of histamine release with human or rabbit antisera has also been described previously.¹² Briefly, an appropriate concentration of antigen (determined by a previous dose-response curve) is mixed with either normal serum (control) or with dilutions of the antiserum made up in normal serum so as to keep the serum concentration constant (experimental), and is incubated for 30 minutes or more at room temperature. The "indicator" cells, from an appropriately allergic donor, are added and the reaction allowed to proceed for 60 minutes at 37° C. The histamine released is assayed, calculated, and the per cent inhibition caused by

the antiserum is derived from the formula $\frac{C-E}{C} \times 100$ in which C is the per cent histamine released in the control tube and E is the per cent histamine released in the experimental tube.

RESULTS

Twenty-three donors were studied by histamine release to ragweed antigen E and Ra.3. Examples of the patterns of sensitivity are shown in Fig. 3. At one extreme were the patients highly sensitive to antigen E, who showed no reactivity to Ra.3, even at concentrations 10³ or 10⁴ times higher (Fig. 3, A). More common were the individuals who reacted to both allergens but were significantly less sensitive to Ra.3. The cells of the patients shown in Fig. 3, B required ten- and one hundredfold more antigen to give 50 per cent histamine release to antigen Ra.3 than antigen E. Other patients were about equally sensitive to the two materials and finally, on the other extreme, were some patients more sensitive to Ra.3. Examples are shown in Fig. 3, C of patients who were three- and tenfold more sensitive to Ra.3 than to antigen E.

The complete series of responses to these to antigens are recorded in Table I, and the sensitivity to Ra.3 is plotted against that to antigen E. in Fig. 4. Of the 23 patients, 3 were more sensitive to Ra.3 than to antigen E, 3 were of about equal sensitivity, and the remainder were tenfold or more less sensitive to Ra.3, including 6 who had no response whatsoever at the highest Ra.3 con-

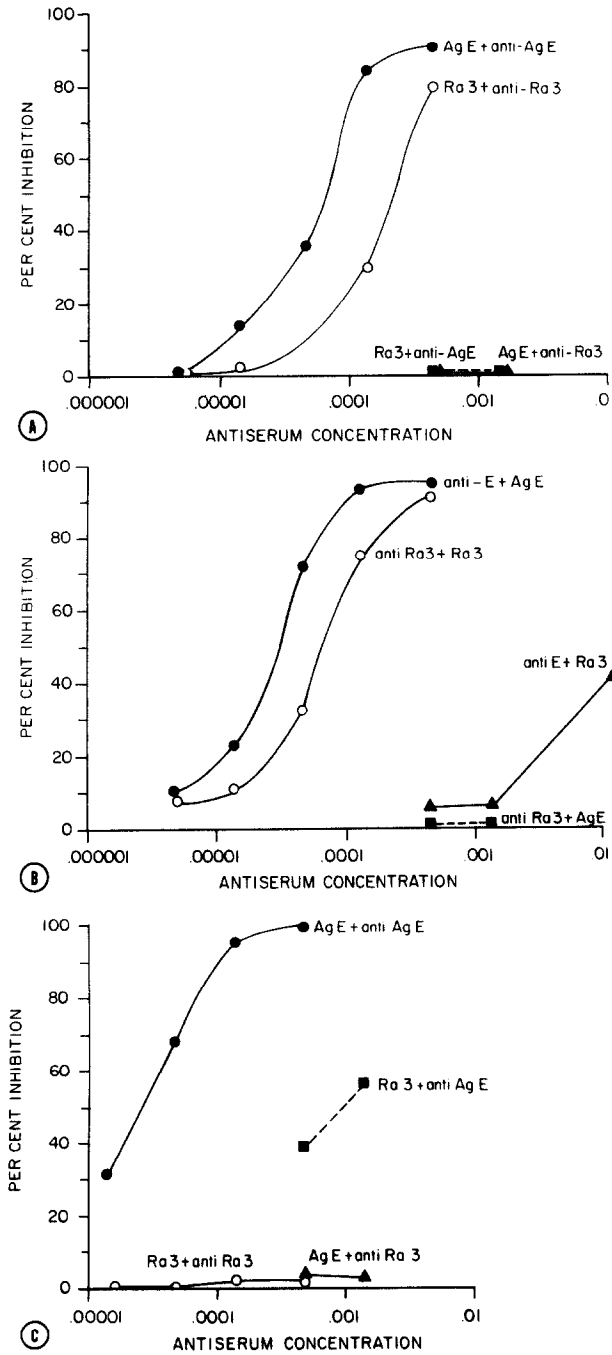


FIG. 7. Inhibition of antigen E and Ra.3 histamine release by rabbit antisera. **A**, Specific inhibition by rabbit antisera to antigens Ra.3 and E. **B**, The same inhibition pattern as **A**, but demonstrating some cross-reactivity between rabbit anti-antigen E and antigen Ra.3. **C**, The failure of anti-Ra.3 to inhibit antigen Ra.3-mediated histamine release together with its inhibition by anti-antigen E. The unusual pattern has been observed in 2 patients.

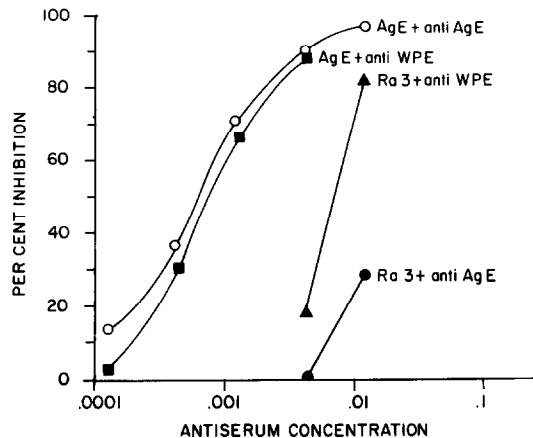


Fig. 8. Inhibition of antigens Ra.3 and E by human antisera to antigen E and whole pollen extract. The human antisera are each pools from 10 allergic patients treated with the appropriate antigen during immunotherapy.

centration employed (usually 1,000 to 10,000 ng. per milliliter). In a series of end point skin titrations of Ra.3 and antigen E on 19 additional individuals, a similar pattern was found: 3 were more sensitive to Ra.3 than to antigen E, 3 were about equally sensitive, and the rest were less sensitive to Ra.3, including 7 who had no response at the highest concentration of Ra.3 used for skin testing (100 ng. per milliliter) (Fig. 5).*

It is clear from the results that there is no constant relationship between the sensitivity of a ragweed-allergic donor to these two antigens, as is the case with cross-reacting allergens such as antigens E and K.

We were also interested in ascertaining whether Ra.3 was a more important allergen in that small group of insensitive individuals who react to whole ragweed pollen extract but are only marginally reactive to antigen E. Although we have not found and, therefore, could not study many such individuals, this does not seem to be the case. An example is the patient whose cell reactivity is shown in Fig. 6. A 50 per cent response to the crude pollen is seen at about 30 ng. per milliliter, while antigen E gave only a 25 per cent response at 4,000 ng. per milliliter. Ra.3 at this same concentration provoked an even smaller, 10 per cent, response.

In order to explore the antigenicity of these two preparations, antisera raised in rabbits against purified Ra.3 and antigen E were used to inhibit the histamine release caused by the homologous and heterologous antigens. The expected pattern was found in most patients in that anti-antigen E inhibited antigen E-induced histamine release but not that caused by Ra.3, while anti-Ra.3 inhibited the response to Ra.3 but not to antigen E (Fig. 7, A). In other patients, the anti-antigen E also caused inhibition of Ra.3 histamine release (Fig. 7, B) but only at an antiserum concentration 10^2 or 10^3 times higher than that needed to inhibit the homologous antigen. In 2 cases an unexpected pattern

*We are grateful to Dr. David Marsh for carrying out these skin tests.

TABLE I. Responses to Ra. 3 and antigen E

Donor	Cell sensitivity* to:		Ratio
	Ra3†	AgE†	
1	4.8	10	0.5
2	10	4.5	2
3	1.2	11	0.1
4	220	0.30	700
5	7.0	2.1	3.5
6	17	1.0	17
7	810	11	70
8	None	5.2	> 1,000
9	None	4.0	> 1,000
10	3.8	0.35	10
11	None	0.33	> 10,000
12	0.44	0.92	0.5
13	10	0.32	30
14	None	1.0	> 1,000
15	None	1.4	> 1,000
16	None	1.6	> 1,000
17	14,000	1.8	10,000
18	45	0.14	300
19	1,600	50	30
20	11%‡	25%‡	—
21	50	3.0	17
22	2.0	0.68	2.9
23	15	1.2	12

*Cell sensitivity is the concentration of antigen required for 50 per cent histamine release.

† $\times 10^{-4}$ μ g per milliliter.

‡Maximal histamine release.

was seen (Fig. 7, C) in which anti-antigen E inhibited antigen E histamine release and, at ten- and fiftyfold higher concentrations, also blocked the response to Ra.3, while the anti-Ra.3 was completely inactive both against the homologous and the heterologous antigens. This was a highly reproducible observation in these 2 individuals, and, while we have not carried out a population study to see how common this phenomenon is, it certainly requires an explanation.

We also explored the inhibition pattern in human sera obtained from allergic donors who had received immunotherapy. In this instance we were limited to anti-antigen E and anti-whole pollen extract (WPE) since Ra.3 has not been used for therapeutic purposes. These two were of interest, however, since if Ra.3 is an independent antigen the anti-WPE should be considerably more effective in blocking it than the anti-antigen E, while the ratios of inhibition by anti-antigen E and anti-WPE against the two antigens should be quite different. A result typical of the 3 donors assayed is shown in Fig. 8. The anti-whole pollen extract is about tenfold more active against E than against Ra.3, and, while the two antisera are equally active in inhibiting antigen E-induced histamine release, the anti-WPE is considerably more effective than the anti-antigen E although, again, the latter has some activity against Ra.3.

DISCUSSION

The data presented in Figs. 3 to 6 and Table I demonstrate that Ra.3 is a distinct allergen that has essentially no allergenic cross-reactivity with the

major ragweed pollen allergen, antigen E. There is no relationship between the sensitivity to the two materials in the 23 patients assayed by histamine release or in the 19 screened by skin testing. In terms of relative activities in different allergic patients, there is more than a 100,000-fold spread. For example, the concentrations of Ra.3 and antigen E required for 50 per cent histamine release in Patient 3 were 1.2 and 11×10^{-4} μg per milliliter, respectively, so that this patient was about ten times more sensitive (ratio = 0.1) to Ra.3 than antigen E. The same figures for Patient 17, at the other extreme, were 14,000 and 1.8×10^{-4} μg per milliliter; this patient was about 10,000 times *less* sensitive (ratio = 10,000) to Ra.3 than to antigen E. In describing similar experiments with ragweed antigen E and K, we developed the argument that cross-reacting allergens show similar patterns of activity.⁷ While it is unnecessary to review this argument, the present data serves to confirm it. At the same time the $> 10^5$ -fold ratio in individuals between Ra.3 and antigen E demonstrates that the Ra. 3 preparation is free from any significant contamination with antigen E.

There are numerous allergens in crude ragweed pollen extract. While E and K are of major clinical importance, for many purposes Ra.3 is of great interest. Its smaller molecular weight makes it easier to study chemically, while the wide range of sensitivity to it in different patients makes it ideal for genetic studies into the patterns of inheritance of responsiveness to different allergens. Ra.3 is probably not the allergen(s) responsible for the response to crude pollen in antigen E-insensitive donors (Fig. 6). Thus, it seems likely that there remain a number of allergens in the crude extract that await identification.

In studying the allergenicity and antigenicity of Ra.3 by inhibiting histamine release with specific antisera, two possible variables are operative. On the one hand, there are the antigens or antigenic determinants seen by the immunized animal (or man) to which the antibodies are made. On the other hand, there are the allergenic determinants detected by the cell-bound IgE antibody on the target cells used as the assay system. The antigenic and allergenic determinants may not be the same. The response expected in these experiments is shown in Fig. 7, *A* and *B*, where specificity is demonstrated. Although there is some anti-Ra.3 activity in the anti-antigen E, this may well be due to contamination in the immunizing material. The data shown in Fig. 7, *C*, in which the anti-Ra.3 has no ability to inhibit Ra.3-induced histamine release, cannot be explained in this way. There are two possible interpretations of this data. Unquestionably a variety of allergenic groups coexist on Ra.3 to which different allergic individuals have a variety of patterns of response. It is possible that the rabbit antisera against Ra.3 lacks antibodies against one of these and that the individuals in question are highly sensitive to that particular allergenic group. Arguing against this hypothesis, however, is the molecular size of Ra.3 and the likelihood that once combined with antibody it would be allergenically inactive either because it was removed from solution or simply on a steric basis. Thus, we have never seen an individual in whom anti-antigen E was not effective in blocking the response to antigen E. It is more likely, perhaps, that the Ra.3 preparation contains a minor allergenic contaminant not detected by a variety of physicochemical techniques⁹ that does

not cross-react with antigen Ra.3 and cross-reacts only weakly with antigen E and that is, finally, inactive in most individuals. While this explanation is similar to the previous one, placing the different determinant on separate molecules is probably more compatible with the observations reported.

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