

In vitro assay of allergenic properties of ragweed pollen antigens

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An in vitro reaction system, based on the release of histamine from isolated leukocytes of allergic donors, has been used to assay the allergenic activity of ragweed pollen antigens. Antigen E was the most potent allergen in tests with leukocyte suspensions of different individuals. The specific activity of other antigens, relative to E, ranged from about 0.5 to 47 per cent. The leukocytes of different individuals vary in their pattern of sensitivity to the several antigens in ragweed pollen. Data are given to show that the sensitivity of this in vitro bioassay procedure exceeds that of the skin test by about twenty times.

Recent chromatographic and immunologic studies have confirmed the presence of numerous antigens in extracts of ragweed pollen (*Ambrosia elatior*).¹⁻⁸ Several of these antigens are now available in highly purified form, but their standardization for diagnostic and therapeutic purposes has been limited by the lack of an adequate bioassay procedure. At present, the assay of these allergens is based on skin testing in humans,^{5, 6} a technique that is poorly reproducible and lacking in precision. It was therefore of interest to ascertain whether the experimental design used in this laboratory for studies of histamine release from human leukocytes⁹⁻¹⁴ might serve as a basis for an in vitro biological assay of human allergens.

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The experiments described below illustrate this attempt. In brief, a suspension of well-washed leukocytes from a ragweed-sensitive donor is incubated with the antigen under study. The outcome of this immune event is expressed in terms of histamine release. The intensity of the response is proportional to the logarithm of the antigen concentration in the range of 20 to 80 per cent histamine release, allowing antigen estimations reproducible to within 20 per cent.¹⁰ Consequently, it might be expected that this procedure would surpass the skin test technique in discriminating between the allergenic activities of different ragweed pollen extracts. The fact that the release of histamine from human leukocytes can be effected with allergens of different specificities^{15, 16} suggests the possible extension of this assay to antigens other than ragweed pollen extracts.

MATERIALS AND METHODS

Antigen preparations

The antigens from ragweed pollen extracts were prepared as described by King and co-workers.^{5, 6} The separation procedure is summarized briefly below to facilitate interpretation of the experimental findings.

Whole pollen is delipidated in ether, extracted in water, and the soluble material is precipitated in 90 per cent saturated ammonium sulfate. This precipitate is dissolved in tris hydrochloride buffer and rendered free of low molecular weight substances by filtration through a Sephadex G-25 column. This procedure yields two fractions, A and B. Fraction B is inactive and is discarded. Chromatography of fraction A on DEAE cellulose yields fractions C and D. The latter is the more active fraction and, when applied to a Sephadex G-100 column, yields fractions I to VI.* Skin tests have shown that fraction IV, which consists almost entirely of a single antigen, designated E, has the highest specific activity. Antigen E can be isolated in four chemical forms by further purification of fraction IV on TEAE cellulose. These forms, designated as IVA, -B, -C, and -D, are proteins of identical molecular weight and amino acid composition but differ in their charge. Analyses of these subfractions by gel diffusion with rabbit antisera have yielded reactions of identity.⁶

The antigens used in this study were stored at a concentration of 100 μ g per milliliter in tris-ACM† buffer at -20° C. The antigen preparations were assayed within a few months after their isolation. However, frozen samples of fraction IV have not decreased in activity after storage at -20° C. for more than three years. Aliquots for daily use were diluted to a concentration of 2 μ g per milliliter in tris-ACM, a fresh sample having been thawed for each experiment.

Antigen assay procedure

One milliliter aliquots of a standardized leukocyte suspension ($1.5 \pm 0.3 \times 10^7$ per milliliter), obtained from a single donor, are placed in a series of reac-

*The source of fractions I_A, I_B, II_A, and II_B is described by King, Norman, and Connell.⁶

†Tris, preset 7.59, 0.025M; NaCl, 0.120M; KCl, 0.005M; HSA, 0.03 per cent; CaCl₂, 6×10^{-4} M; MgCl₂, 1×10^{-3} M.

tion tubes. The antigens to be tested are added in 3 ml. volumes, in dilutions selected to yield a release of histamine in the partial range of response, i.e., 20 to 80 per cent. Since the cells of different donors may vary in their responsiveness to ragweed antigens over a concentration range of four orders of magnitude, preliminary titrations are required to localize the sensitivity of each cell suspension. The cells and antigen are mixed and incubated at 37° C. for 60 minutes with periodic agitation. The mixtures are then centrifuged and the cell-free supernates assayed for liberated histamine.^{16, 17} Duplicate cell aliquots are lysed with perchloric acid to estimate the total histamine content of the cells under study.

EXPERIMENTAL RESULTS

Comparative histamine-releasing capacity of different ragweed pollen antigens

Fractions A and IV. Initial experiments were performed with these two materials, representing a crude and a purified preparation. Fraction A contains the water-soluble material precipitated by 90 per cent saturated ammonium sulfate. It may be considered as a mixture of most, if not all, of the water-soluble protein antigens in ragweed pollen, wherein antigen E represents only 6 per cent of the total protein.⁶ In contrast, fraction IV is a purified derivative of fraction A, consisting of about 87 per cent antigen E (see above). Skin tests showed that of more than 250 individuals who reacted to fraction A, only two failed to give positive skin tests with fraction IV.⁶ In all other individuals fraction IV was more potent than the crude, parent material, fraction A.

To investigate the relationship between fractions A and IV, leukocyte suspensions from donors with ragweed allergy were divided into two portions, for testing with each fraction. Two illustrative dose-response curves are seen in Fig. 1. The graph on the right indicates the results obtained with the leukocytes

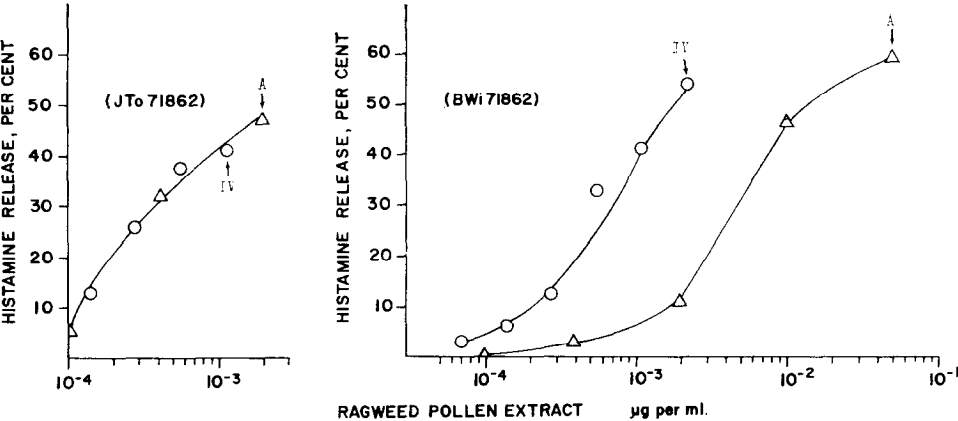


Fig. 1
Histamine release by ragweed pollen extracts A and IV. Comparative responses of leukocytes from two ragweed-sensitive donors.

	<i>JTo</i>	<i>BWi</i>
Histamine available, µg	0.43	0.24
Number white blood cells	2×10^7	1.6×10^7

of one individual (*BWi*). In this case, the amount of fraction *A* required for a given response exceeded that of fraction *IV* by a factor of about 10. The results with the cells of another donor (*JTo*) are shown in the left graph of Fig. 1, where it can be seen that fractions *A* and *IV* were of identical biological potency. Similar results were obtained on retesting the cells of these two individuals with the same preparations after an interval of some weeks. In tests with the cell suspensions of six other donors, each exhibited a unique ratio with respect to the relative activities of fractions *A* and *IV*, although all fell within the range set by *BWi* (1:10) and *JTo* (1:1), as shown in Fig. 1. The actual ratios of fractions *A* to *IV* required for 50 per cent histamine release were: 10.5, 9.3, 9.1, 3.8, 3.2, 2.8, 2.0, and 1.1. It may be reasoned that if fraction *A* contained one or more allergens, all of which had been concentrated to an equal extent in fraction *IV*, a constant ratio of specific activity (i.e., *A:IV*) should have been found in assays with leukocytes from different allergic individuals. Since this ratio is variable, it follows that leukocytes from different donors vary in their content and/or specificity of immunoglobulins capable of releasing histamine upon interaction with a given set of ragweed pollen antigens. This finding is fully consistent with the fact that ragweed pollen contains multiple biologically active antigens. It is also in agreement with the varying patterns of skin reactions observed in individuals tested with the same panel of antigens.^{5, 6} Further evidence along these lines emerged from experiments with the purified fraction *D* and its derivatives.

Fractions I to V, and D. Aliquots of cells obtained from a single blood sample of each allergic donor were titrated with eight antigen preparations.

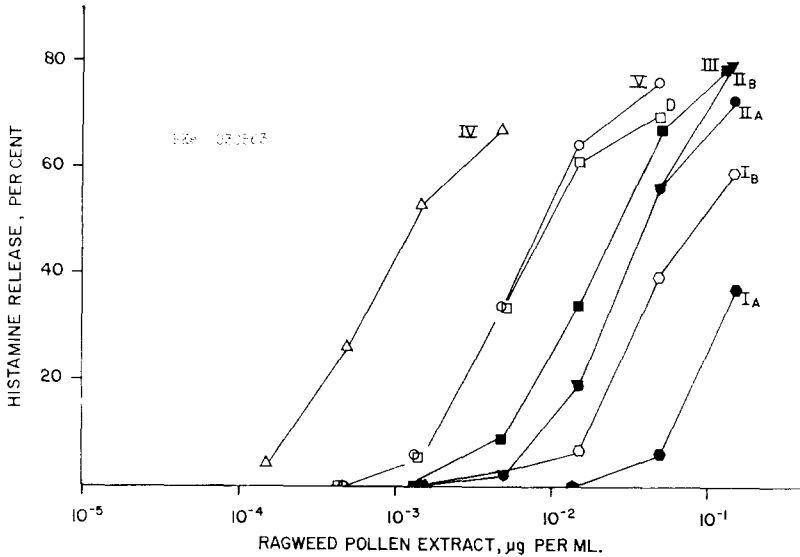


Fig. 2

Dose response relationships of ragweed extract fraction *D* and its subfractions, *I* to *V*.
Number white blood cells, 1.6×10^5 .
Histamine available, 0.37 µg.

The results of a typical titration are presented in Fig. 2, where it may be seen that the biological activity of these fractions varies more than one hundred fold on a weight basis. The slopes of the log dose-response plots in Fig. 2 are roughly similar in the partial range of response, suggesting that the activity of these fractions is comparable in terms of the weight of antigen necessary to release 50 per cent of the cellular histamine store, i.e., specific activity. This comparison is shown for three individuals in Table I. Since the cells of different individuals vary in sensitivity, the comparison is also made in terms of relative activity, arbitrarily setting the activity of fraction IV at 100. These data demonstrate considerable similarity in the pattern of response to these fractions by the three cell suspensions. Taken in their entirety, the results obtained with the leukocyte suspensions parallel the skin reactions elicited with the same antigens (see Fig. 7 of King and Norman⁵). In both studies, fraction IV emerges as the most potent allergen; fractions V and D also exert considerable allergenicity. The allergenicity of fraction D is to be expected since fraction IV is derived from D which contains 19 per cent antigen E.⁶ The low allergenicity of the other fractions might have been attributed to contamination with antigen E. However, the differing patterns of response with individual cell suspensions, as is particularly apparent in the case of fraction V, suggest that this is not the explanation.

As noted above, antigen E represents 87 per cent of the material in fraction IV, and is the substance apparently responsible for its allergenic activity.⁶ Assays were, therefore, conducted with fractions IV_B and IV_C, as well as with a less homogeneous preparation, IV_D. Fractions IV_B and IV_C are immunologically indistinguishable preparations of antigen E which contain less than 5 per cent of other pollen antigens⁶ (see Fig. 4 of King, Norman, and Connell⁶). These fractions were assayed with the cells of three allergic donors. A typical experiment is shown in Fig. 3. Fractions IV_B and IV_C had the same activity, requiring 1.0 and 1.1×10^{-3} μ g per milliliter, respectively, to cause 50 per cent

Table I. *The concentration, in nanograms per milliliter, of fraction D and its derivatives required to cause 50 per cent histamine release from the leukocytes of three allergic individuals. The potency of each fraction is presented in terms of per cent activity relative to IV*

Fraction	Cell donor RKe		Cell donor FGI		Cell donor BWi	
	Concentration of antigen required for 50% release ($\times 10^{-9}$ Gm.)	% activity relative to IV	Concentration of antigen required for 50% release ($\times 10^{-9}$ Gm.)	% activity relative to IV	Concentration of antigen required for 50% release ($\times 10^{-9}$ Gm.)	% activity relative to IV
IV	1.3	100	0.045	100	0.47	100
V	9.0	14	0.12	40	1.0	47
D	10	13	0.45	10	1.2	40
III	27	4.8	0.75	6.0	9.0	5.3
IIA	41	3.2	1.5	3.0	8.5	5.5
IIB	41	3.2	2.0	2.5	24	2.0
IB	90	1.4	3.5	1.3	36	1.3
IA	220	0.6	1.9	2.4	110	0.43

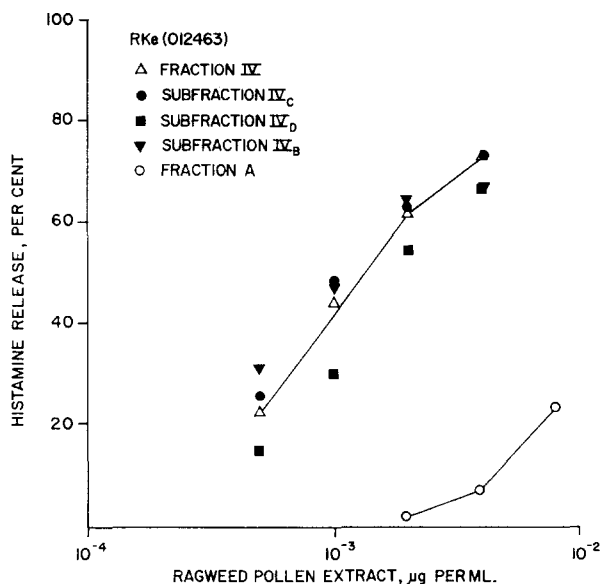


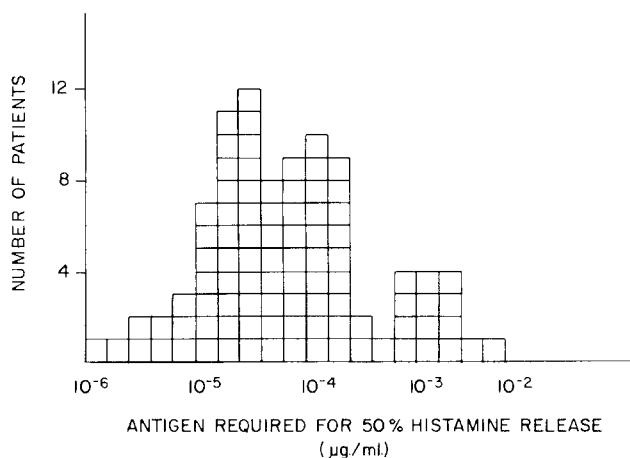
Fig. 3

Dose response relationships of ragweed extract fraction *IV*, its subfractions, and fraction *A*.
Number white blood cells, 2.0×10^7 .
Histamine available, $0.42 \mu\text{g}$.

histamine release with this leukocyte suspension. The parent material, *IV*, was somewhat less active, requiring $1.3 \times 10^{-3} \mu\text{g}$ per milliliter, while $2.0 \times 10^{-3} \mu\text{g}$ per milliliter of *IV_D* was required for a 50 per cent response. With the cells of another individual, the quantities required for 50 per cent histamine release were 1.1, 1.2, 1.5, and $1.9 \times 10^{-5} \mu\text{g}$ per milliliter for fractions *IV_B*, *IV_C*, *IV*, and *IV_D*, respectively. The cells of a third donor also yielded similar ratios of comparative activities for these fractions. These assays indicate the degree of definition that may be achieved with this technique and allow the conclusion that fractions *IV_B* and *IV_C* are more potent than *IV* which, in turn, is more active than *IV_D*. Within the limits of the experimental error, it appears that the activity ratios of *IV_B* and *IV_C*:*IV*:*IV_D* are the same in the three individuals tested. The constancy of these ratios for a larger group of cell donors remains to be verified. It is pertinent, however, that these results again bear close resemblance to the skin test data (see Fig. 9 of King, Norman, and Connell⁶). Moreover, the leukocyte histamine release data compared favorably with the estimations of antigen E in these fractions. Quantitative immunodiffusion analyses established that the concentration ratios of antigen E in these fractions are 1:1:0.9:0.5 for *IV_B*, *IV_C*, *IV*, and *IV_D*, respectively. The corresponding ratios for the histamine releasing potencies of these antigens are 1:0.9:0.75:0.5.

Sensitivity

The histogram in Fig. 4 indicates the distribution of leukocyte sensitivity to fraction *IV* in assays of 92 patients, two thirds of whom had never received

**Fig. 4**

The quantity of ragweed pollen fraction IV required for 50 per cent histamine release from the leukocytes of 92 ragweed-sensitive donors.

ragweed antigens parenterally for diagnostic or therapeutic purposes. Over half of these donors required between 1 and 10×10^{-5} μg per milliliter to achieve 50 per cent histamine release, and the range was from 10^{-6} to slightly less than 10^{-2} μg per milliliter. This may be compared with the skin testing of fraction IV, the results of which showed that in about two thirds of 159 donors, minimal reactivity was noted with the administration of 10^{-4} to 10^{-6} μg of antigen. (Since 0.05 ml. was injected, the actual concentrations of the solutions were 2×10^{-3} to 2×10^{-5} μg per milliliter.) The amounts of antigen required for a maximal skin response were ten to one hundred fold higher. These data suggest that release of histamine from reactive leukocytes is a somewhat more sensitive technique than skin testing.

Reproducibility

The data in Fig. 5, *A* and *B* bear on the reproducibility of the histamine release assay when the same leukocytes are used at different times. Two dose-response curves for one set of cells are compared in Fig. 5, *A*. The 50 per cent response values are virtually identical and are well within the limits of experimental error (± 10 per cent). Greater variation is seen in replicate assays carried out over a longer period of time (Fig. 5, *B*). At the 50 per cent intercept, the values ranged from 7 to 25×10^{-5} μg of fraction IV per milliliter in 12 separate experiments carried out over a period of more than three years with the cells of a single donor, RDa. In 34 individuals whose dose-response curves were repeated 5 to 6 times over a 9 month period, there was no statistically significant change noted in the antigen required for 50 per cent histamine release, although there was a tendency of the cells of individuals who had been desensitized to require somewhat more antigen. The possible effects of parental pollen therapy on cell sensitivity are under study.¹⁸

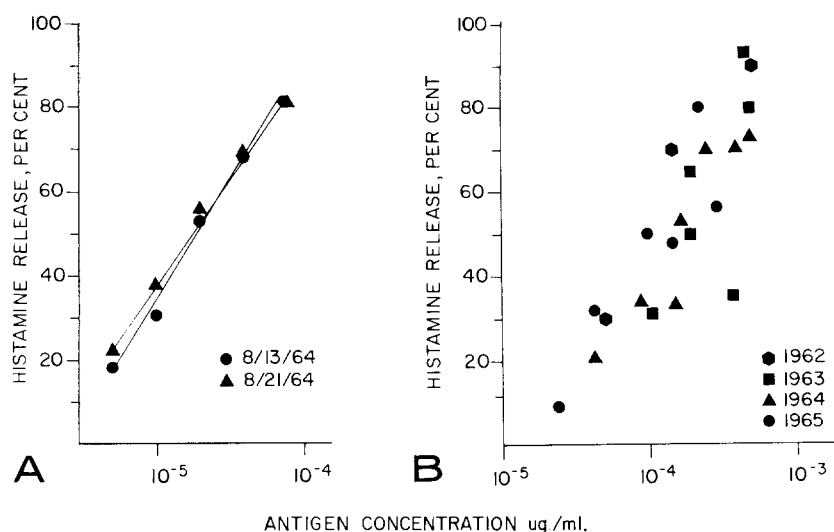


Fig. 5

A, Dose-response curves performed with the leukocytes of one allergic donor (LML) on the dates indicated.

B, Dose-response curves obtained in 12 separate experiments performed over a 3 year period with the leukocytes of a single allergic donor (RDa).

The antigen used in each experiment was ragweed pollen fraction IV.

DISCUSSION AND SUMMARY

The experiments described above illustrate the application of an *in vitro* model of human allergic reactions to the bioassay of allergenic preparations. The assay mimics *in vivo* events with respect to the liberation of a vasoactive amine by physiologic quantities of the antigens which mediate ragweed hypersensitivity (Fig. 4). As noted, the same patterns of response follow the use of several ragweed pollen extracts of varying degrees of purity, in human skin tests and in the *in vitro* histamine release from human leukocytes. The leukocyte reaction system provides greater resolution than the skin test, exceeding the latter by about twentyfold. The use of a standardized suspension of leukocytes also tends to facilitate test replication and reproducibility of results (Fig. 5, *A* and *B*).

The bioassays performed with the various fractions isolated from ragweed pollen confirm the earlier skin testing data in demonstrating that fraction IV (antigen E) has the greatest potency. Moreover, the relative activities of the other fractions are readily established, exemplifying the utility of this *in vitro* system in antigen purification procedures (Table I). The skin test data permitted similar conclusions but lacked the quantitative precision necessary for antigen standardization.

The data in Table I and in Figs. 1 and 2 indicate that a unique pattern of sensitivity to different ragweed pollen antigens characterizes the leukocytes of each individual, a conclusion that could not be drawn from the skin test data with the same degree of validity. The therapeutic implications of these findings

are not yet clear, but the results achieved to date encourage the continued utilization of the in vitro leukocyte reaction system for studies of human pollinoses. As one example, combined laboratory and clinical studies have shown that the degree of leukocyte sensitivity to antigen E is well correlated with symptom severity during the ragweed season.¹⁸

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