

PRIMARY INTERACTION BETWEEN I^{131} LABELED RAGWEED POLLEN AND ANTIBODIES IN THE SERA OF HUMANS AND RABBITS

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SEVERAL in vitro immunologic techniques such as the precipitin reaction,¹ agglutination of antigen-coated collodion particles,² complement fixation,³ and hemagglutination⁴ have been used to study the antibodies to pollen antigens in sera of humans and animals. All of these tests have in common the fact that they are dependent upon a secondary reaction occurring subsequent to a primary antigen-antibody interaction.⁵ Inasmuch as the secondary reactions do not necessarily reflect the total extent of the primary interactions, there is a need for a method which measures the primary binding of pollen antigens by antibody in order to compare such results with secondary manifestations in vivo or in vitro.

The present study describes what appears to be a feasible approach to the problem of measuring the primary interaction between ragweed pollen antigens and circulating anti-ragweed antibodies. It is based on the observation that ammonium sulfate will precipitate I^{131} labeled antigen-antibody complexes in some immune systems when the antigens in question are soluble in the same concentration of ammonium sulfate.⁶ Utilizing a partially purified I^{131} labeled ragweed pollen extract (I*RW) as the antigen and 40 per cent saturated ammonium sulfate (SAS) to precipitate I*RW-globulin complexes, the method appears to quantitatively and reproducibly measure the specific binding between at least some antigenic groups associated with ragweed pollen and antibodies in sera from humans and rabbits that have been previously immunized with ragweed pollen extract.⁷

MATERIALS AND METHODS

Diluents, Reagents and General Procedures.—The borate buffer (pH 8.3) and saturated ammonium sulfate used in this experiment were prepared as previously described.⁶ The saline-borate buffer was a mixture of 9 parts of 0.85 per cent NaCl in distilled water and 1 part of borate buffer.

All dialyses, incubation periods, fractionations, and extractions were carried out at 4° C. unless otherwise noted.

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Preparation of Ragweed Pollen Extracts.—

1. *Ragweed pollen extract used for clinical hyposensitization:* Defatted and dried dwarf and giant ragweed pollen were separately suspended in Coca's fluid at a 5 per cent concentration and refrigerated over night at 4° C. The suspensions were then passed through a Seitz filter and the filtrates were tested for bacterial sterility. An equal volume of glycerin was added to each of the ragweed extracts (dwarf and giant) and these in turn were combined so that the final glycerinated mixture contained 400 γ ragweed pollen nitrogen per milliliter.* This extract, in varying dilutions, was used for clinical hyposensitization.

2. *Ragweed pollen extract used for rabbit immunization:* Ten grams of defatted ragweed pollen (equal parts of giant and dwarf) were extracted for 24 hours in 200 c.c. of Coca's fluid. The mixture was filtered and an equal volume of borate buffer was added to the filtrate. The resultant mixture was dialyzed against frequent changes of saline-borate buffer for 48 hours. This dialyzed mixture was concentrated by pervaporation so that the final mixture contained 430 γ ragweed pollen N per milliliter.

3. *Ragweed pollen extract used for inhibition tests:* The ragweed pollen extract prepared for rabbit immunization was diluted with an equal volume of borate buffer and precipitated in the presence of 50 per cent SAS. The supernatant was dialyzed against a saline-borate buffer for 24 hours. This final mixture, when diluted 1:10 in borate buffer, was used as the unlabeled ragweed inhibitor and contained 2 γ N per milliliter. No polyvinyl pyrrolidone (PVP) or glycerine was used in its preparation.

Preparation of I^{131} Labeled RW Extract (I^*RW).—

The glycerinated ragweed extract used for clinical hyposensitization was dialyzed against frequent changes of the saline-borate buffer containing 1 per cent PVP for 48 hours to clear the glycerin. Subsequently, the sac contents were dialyzed against saline-borate buffer without PVP to remove any PVP fragments. The dialyzed extract was diluted 1:2 with borate buffer, an equal volume of saturated ammonium sulfate added, the mixture incubated for 30 minutes prior to centrifugation at 2,200 r.p.m. for 30 minutes, and the supernatant decanted and dialyzed against saline-borate buffer until the dialysate was free of sulfate ions. The dialyzed supernatant was then concentrated by pervaporation to the original volume and divided into 1.0 ml. aliquots for storage in the deep freeze at -20° C. until used.

The final extract contained 400 γ ragweed pollen N per milliliter and a 0.5 ml. sample was iodinated with 40 millicuries of I^{131} by the method of Talmage and co-workers.⁸ After iodination, the labeled material was passed through an Amberlite resin column† and then dialyzed against multiple changes of saline-borate buffer for 24 to 36 hours to remove I^{131} not bound to RW extract. The nondialyzable portion contained 15 γ ragweed pollen N per milliliter.

The final step in the preparation of the test antigen was to eliminate any fractions which would precipitate in the presence of normal serum and 50 per

*Obtained from Allergy Laboratory of Montefiore Hospital, Pittsburgh, Pa.

†Resikit, E. R. Squibb & Sons, New York, New York.

cent SAS. Details of this procedure were as follows: (a) 20 ml. of 1:10 normal human serum (or normal rabbit serum) was mixed with 16 ml. of borate buffer and 4 ml. of the iodinated dialyzed ragweed pollen extract; (b) the mixture was refrigerated overnight, 40 ml. of SAS added, and refrigerated again for 30 minutes; (c) the mixture was centrifuged for 30 minutes at 2,200 r.p.m. and the supernatant decanted; and (d) the supernatant was dialyzed against saline-borate buffer until the dialysate was free of sulfate ions.

Unless otherwise stated, the supernatant was then diluted 1:10 with 1:100 normal rabbit serum (NRS) in borate buffer and was designated as I*RW. An I¹³¹ labeled ragweed pollen nitrogen determination could not be performed on this final product because of the serum protein nitrogen added but, on the basis of dilution and volumetric changes during dialysis, the final solution probably contained approximately 0.04 γ I*RW N per milliliter.

Preparation and Selection of Sera.—

1. *Rabbit antisera:* Ten milliliters of aqueous ragweed pollen extract, prepared for rabbit immunization as described above, was added to 8.5 ml. of Bayol F and 1.5 ml. of Arlacel. The mixture was emulsified by mixing at full speed in a Servall Omnimixer for 1 minute. Mature male New Zealand white rabbits were injected with a total of 3 ml. (645 γ ragweed pollen N) of the emulsified antigen into 6 subcutaneous sites over the back. One month later, the rabbits were given 0.5 ml. (215 γ ragweed pollen N) of the aqueous extract intravenously via the marginal ear vein and the adjuvant immunization repeated 4 days later. Sera were obtained 2 months subsequent to the original antigen-emulsion injection.

2. Human sera:

A. Reference standard serum (RSS). The serum from an individual with a negative history for clinical allergy attributable to ragweed pollen was selected for use as a reference standard in all experimental procedures. The donor for this serum had a negative intradermal test to 0.02 ml. of a 10.0 γ N per milliliter ragweed pollen extract, and was skin tested on one occasion 7 to 30 days before several bleedings.

B. Nonallergic sera. Sera were obtained from 100 patients at the Presbyterian Hospital and Falk Clinic of the University of Pittsburgh. In this group there were no patients with a history of clinical sensitivity to ragweed pollen, asthma, rhinitis, or allergic disease. In addition, patients being treated with bronchodilators, steroids, or antihistamines, and those with a positive skin test to a 10.0 γ N per milliliter test solution of ragweed pollen extract were eliminated from this study. Eighty-seven patients of this group were hospitalized with a variety of acute and chronic medical diseases while the 13 clinic patients were medically asymptomatic.

C. Ragweed-sensitive sera. Sera were obtained from ragweed-sensitive patients, both treated and untreated, who attended the Immunology Clinic of the Falk Clinic of the University of Pittsburgh, the Allergy Clinic of Montefiore Hospital of Pittsburgh, and the Division of Allergy and Infectious Diseases of the Johns Hopkins Hospital, or from those seen in private practice. The

treated patients had received at least 5 months of aqueous ragweed pollen injection therapy or one injection of a water-in-oil emulsion of a ragweed pollen extract a month prior to the collection of serum.

*Basic Experimental Procedure to Test for the Presence of Anti-I*RW.—*

Human and/or rabbit antiserum were diluted 1:10 in borate buffer and duplicate 0.5 ml. aliquots incubated 18 to 24 hours at 4° C. with 0.5 ml. I*RW. In addition, control tubes were prepared with the same I*RW and 1:10 normal serum from the same species. After incubation, 1 ml. of 80 per cent SAS in distilled water was added and the tubes immediately shaken in a vigorous fashion so as to insure complete mixing of the heavy ammonium sulfate solution with the less dense I*RW-sera mixtures. An additional incubation period of 30 minutes, still at 4° C., was allowed for precipitation of globulins and any I*RW-globulin complexes. Following centrifugation for 30 minutes at 2,200 r.p.m., the supernatant was decanted from the precipitate and the precipitate washed with 40 per cent SAS in borate buffer. The precipitate was then counted in a well-type gamma scintillation counter. The calculation of the per cent precipitated was determined by the ratio of the counts per minute in the precipitate to the total counts per minute of the I*RW initially added.

For each experiment involving human serum, the reference standard serum described above was also tested.

EXPERIMENTAL PROCEDURES AND RESULTS

1. Retention of the Biologic Activity of Ragweed Pollen Extract After Labeling With I^{131} .

To determine whether the iodination procedure itself had altered the biologic activity of the ragweed pollen extract, the iodinated extract was allowed to undergo radioactive decay for 2 months, sterilized with a Sweeney filter, and diluted with buffered saline so that the final extract contained 1.5 γ N per milliliter. Intradermal tests with 0.02 ml. of this iodinated extract, a buffered-saline control, and unlabeled ragweed pollen extract containing 1.0 γ N per milliliter were performed on an untreated ragweed-sensitive individual and on an individual with no history attributable to ragweed pollen. The iodinated extract elicited a wheal-and-flare response in the ragweed-sensitive individual which was comparable to the response with the unlabeled extract. Both the labeled and unlabeled extracts failed to elicit an intradermal response in the nonsensitive individual. The buffered-saline control solution caused no reaction in either the sensitive or control individual.

*2. Precipitation of I*RW by Normal and Antiragweed Rabbit Sera in the Presence of 40 and 50 Per Cent Saturated Ammonium Sulfate.*

Duplicate samples of normal rabbit serum and of antiragweed sera were prepared according to the basic experimental plan by precipitation with an equal volume of 80 per cent SAS and washing the precipitate with 40 per cent SAS. Another duplicate set of these sera were treated in the same manner except that they were precipitated in an equal volume of SAS and washed with 50 per cent SAS. In Table I, it can be seen that at final concentrations of both 40 and

50 per cent SAS, the antisera bound approximately 50 per cent more of the total I*RW added than did the normal rabbit serum. It can also be seen that the per cent of I*RW precipitated by normal rabbit serum increased from 6.3 to 26.4 per cent when the concentration of SAS was increased from 40 to 50 per cent. Under the same conditions, however, the per cent I*RW precipitated by each antiragweed serum was also increased approximately 20 per cent. Subsequent sections of this report will demonstrate that the difference between the per cent precipitated by the antiserum and the per cent precipitated by the normal serum was due to binding of I*RW by antibody, and, since this difference remained unchanged with the 10 per cent increase in SAS concentration, the additional amounts of I*RW precipitated at 50 per cent SAS by both normal and antiragweed sera were probably due to the solubility characteristics of the I*RW per se. Evidence will be presented below (Table IV) which indicates that even the 6 per cent I*RW precipitated by normal serum after the addition of 40 per cent SAS was due to the inherent insolubility of I*RW in 40 per cent SAS. Because of these considerations, the 40 per cent SAS was used to precipitate the I*RW-antibody complexes in all subsequent experiments.

TABLE I. THE PER CENT OF I*RW PRECIPITATED BY NORMAL AND ANTIRAGWEED RABBIT SERA IN THE PRESENCE OF 40 AND 50 PER CENT SAS

RABBIT SERUM (1-10)	PER CENT OF 0.02 γ I*RW N/ML. PRECIPITATED				
	50% SAS		40% SAS		50% TOTAL MINUS 40% TOTAL
	TOTAL	TOTAL MINUS NRS	TOTAL	TOTAL MINUS NRS	
Normal (NRS)	26.4		6.3		20.1
Ragweed injected					
1030-8	75.9	49.3	56.4	50.1	19.5
1031-8	74.4	48.0	55.6	49.3	18.8

*3. Effect of I*RW Concentration Upon the Precipitation of I*RW by Human Sera in the Presence of 40 Per Cent SAS.*

Whereas Table I demonstrated that it was possible to detect antibody in the serum of rabbits immunized with ragweed pollen extract, Table II shows it was possible to detect increased binding of I*RW by sera from certain selected humans also immunized with ragweed pollen extract. The fact that increased capacity to bind I*RW was not detected in the sera of nonallergic patients or of ragweed-sensitive individuals who had not received parenteral ragweed pollen extract therapy can also be seen in Table II and will be further shown in Table V.

In addition, Table II also illustrates that the concentration of I*RW was a critical determinant in detecting antibody in the sera from humans immunized with ragweed pollen extract. A tenfold diminution in the concentration of I*RW from 0.2 γ to 0.02 γ N per milliliter elicited a significant increase in the per cent of I*RW bound by the sera of the treated ragweed-sensitive group while the per cent of I*RW bound by the other two groups and by the controls remained essentially unchanged. Although further dilution of I*RW further increased the per cent of I*RW bound by the same antisera, the 0.02 γ I*RW N per milliliter concentration was used in the subsequent studies because the I¹³¹ activity of the more dilute solutions was too low for accurate counting.

4. Effect of Antiserum Dilution Upon the Precipitation of I*RW in the Presence of 40 Per Cent SAS.

The effect of antiserum-dilutions upon the per cent of 0.02 γ I*RW N per milliliter precipitated by two representative antisera is presented in Fig. 1. The sera were diluted 1:10 in borate buffer and subsequent dilutions were made in 1:10 normal rabbit serum-borate buffer. It can be seen that the per cent of I*RW precipitated decreased with antiserum dilution, but that the increased precipitation of I*RW was still demonstrable with as little as 0.5 ml. of a 1:100 ragweed-injected human antiserum or 0.5 ml. of a 1:2000 ragweed-immunized rabbit antiserum.

5. Failure of Antisera Against Unrelated Antigens to Precipitate Increased Amounts of I*RW.

A group of antisera directed against apparently unrelated antigens were studied to learn whether increased amounts of I*RW would be precipitated by

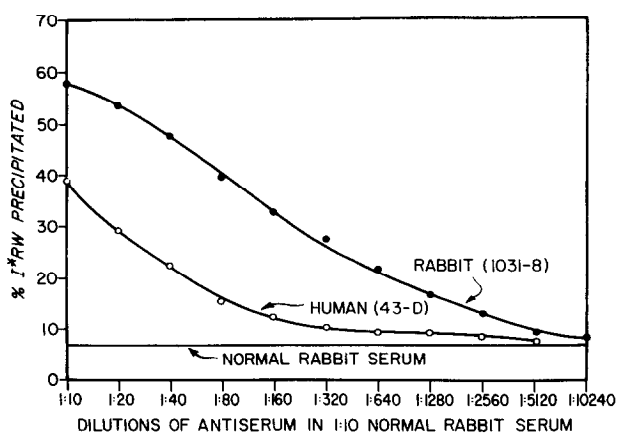


Fig. 1.—The effect of antiserum concentration upon the per cent of I*RW precipitated by 40 per cent SAS.

TABLE II. THE EFFECT OF I*RW CONCENTRATION ON THE PRECIPITATION OF I*RW BY HUMAN SERA

HUMAN SERUM 1:10	PER CENT PRECIPITATED WITH	
	0.2 γ I*RW N/ML.	0.02 γ I*RW N/ML.
Reference standard	8.3	9.4
Ragweed-sensitive		
# 9	9.1	7.1
# 11	7.3	8.9
# 12	6.2	6.7
# 14	7.4	6.9
Treated ragweed-sensitive		
# 8	12.7	18.8
# 17	11.9	19.3
# 22	11.8	16.7
# 32	20.1	31.9
Chest diseases, nonallergic		
# 15	6.0	5.5
# 28	6.8	7.6
# 30	7.0	6.1

these antisera in some nonspecific fashion. Accordingly, Table III demonstrates that high titer rabbit antisera against bovine albumin (BSA) and globulin, human globulin, and Type 12 streptococcal M-protein did not precipitate more I*RW than did normal rabbit sera. Similarly, sera obtained from patients in whom immunologic mechanisms might be operative were also studied and they precipitated no more I*RW than did the human reference standard serum.

TABLE III. THE FAILURE OF UNRELATED ANTISERA TO PRECIPITATE INCREASED AMOUNTS OF I*RW

TEST SERUM	NUMBER OF SAMPLES	PER CENT OF 0.02 γ I*RW N/ML. PRECIPITATED
<i>Rabbit</i>		
Normal (NRS)	26	8.4 $\pm$ 1.9*
Anti-ragweed		
1030-8	1	56.4
1031-8	1	55.6
Antibovine globulin	1	9.5
Antibovine albumin	1	14.5
Antihuman globulin	1	7.9
Antistreptococcal M-protein	1	11.0
<i>Human</i>		
Reference standard	1	11.0
"Normal" nonallergic	99	8.1 $\pm$ 1.3*
Penicillin hypersensitivity	5	9.1 $\pm$ 1.4*
Serum sickness	1	13.2
Egg sensitivity	4	8.4 $\pm$ 1.3*
Hyperglobulinemia	1	11.1

*1 Standard deviation.

6. Specific Inhibition of the I*RW-Antiragweed Interaction by the Addition of Unlabeled Ragweed Pollen Antigens.

The specificity of the I*RW-antiragweed antibody interaction was also studied by means of an inhibition test utilizing unlabeled ragweed pollen extract and unlabeled BSA and streptococcal M-protein. The experimental procedure for the inhibition test was a slight modification of the routine method of testing for anti-I*RW. Specifically, 0.5 ml. of borate buffer or 0.5 ml. of the unlabeled antigen in borate buffer was incubated with 0.5 ml. of the 1:10 test serum at 37° C. for 2 hours prior to the addition of 0.5 ml. of I*RW. The mixture was shaken, incubated for 18 hours at 4° C., and 1.5 ml. of 80 per cent SAS was then added to each tube. The rest of the procedure was identical to that previously described above for ammonium sulfate precipitation.

The data obtained by this inhibition technique are presented in Table IV. It is apparent that 1 γ N of unlabeled ragweed pollen extract markedly inhibited the binding of I*RW when the unlabeled ragweed pollen was added prior to the I*RW. In contrast, 100 γ N of Type 12 streptococcal M-protein and 80 γ N of bovine serum albumin did not interfere with the binding of I*RW under the same conditions. It is also apparent in Table IV that the 1 γ unlabeled ragweed pollen extract N did not depress the per cent I*RW bound by normal serum controls. This lack of effect is further evidence that the I*RW precipitated in the presence of 40 per cent SAS was not the result of specific binding, but was more likely an expression of the innate solubility characteristics of I*RW in ammonium sulfate.

TABLE IV. THE SPECIFIC INHIBITION OF THE I*RW-ANTIRAGWEED INTERACTION BY THE ADDITION OF UNLABELED RAGWEED ANTIGENS

SERA	PER CENT OF 0.02 γ I*RW N/ML. PRECIPITATED UPON ADDITION OF:			
	DILUENT (0.5 ML.)	RAGWEED (1 γ N/0.5 ML.)	STREP. M12 (100 γ N/0.5 ML.)	BSA (80 γ N/0.5 ML.)
<i>Controls</i>				
NRS	11.0	10.5		
Reference standard	11.5	10.5		
RW-Immunized				
Rabbit				
1030-8	39.1	13.0	39.2	39.3
1031-8	40.7	12.8	40.4	40.0
Human				
L18	33.6	8.3	33.7	34.0
L14	35.8	8.9	37.3	36.0
43D	38.1	9.8	38.0	37.5

7. *Precipitation of I*RW by Sera From Normal, Ragweed-Sensitive, and Ragweed-Treated Sensitive Humans After the Addition of 40 Per Cent SAS.*

In Table II it was shown that the increased I*RW precipitation could be demonstrated in the sera of 4 selected ragweed-sensitive patients following parenteral administration of ragweed pollen extract. It was necessary to select the sera presented in Table II because increased I*RW binding was not detected in all sera from the individuals immunized with ragweed pollen extract. It was, therefore, of interest to know how many of a group of ragweed pollen-immunized individuals would indeed produce anti-I*RW as measured by this method. Furthermore, the question arose as to whether the sera from ragweed-sensitive individuals as a group bound more I*RW than did the sera from a group of nonallergic individuals. Accordingly, Table V compares the I*RW precipitation by sera from 99 nonallergic individuals, 29 ragweed-sensitive individuals before hyposensitization, and 80 ragweed-sensitive individuals after parenteral injection of ragweed pollen extract.

TABLE V. THE PRECIPITATION OF I*RW BY SERA FROM NORMAL, RAGWEED-SENSITIVE, AND RAGWEED-TREATED SENSITIVE HUMANS

SERA	NUMBER OF SAMPLES	PER CENT OF 0.02 γ I*RW N/ML. PRECIPITATED		
		AVERAGE	RANGE	S.D.
Reference standard	1	11.0		
Nonallergic	99	8.1	5.0-12.5	1.3
Ragweed-sensitive (untreated)	29	9.8	7.1-13.2	1.6
Ragweed-sensitive (immunized)	25	11.8	7-14.9	1.9
	23	18.6	15-20.9	1.9
	32	26.7	21-43	4.9
	80	19.7	7-43	3.4

It can be seen that the sera of untreated ragweed-sensitive individuals precipitated no more I*RW than did the sera from the nonallergic group. These two groups were similar with respect to the average, the range, and the standard deviation of the per cent I*RW precipitated. The data from these control groups is presented in its entirety except for the results obtained with

one serum from a nonallergic patient who had received 100 mg. digitalis leaf per day for 15 months. This serum precipitated 17 per cent of the I*RW, but the result was excluded from the data of the nonallergic group because animals^{9, 10} and man¹¹ can be immunized via the oral route and subsequent experiments have shown a small degree of shared antigenicity exists between digitalis leaf extract and ragweed pollen extract.¹²

The data in Table V concerned with the sera from the immunized ragweed patients has been subdivided into three groups according to the amount of I*RW precipitated after the addition of 40 per cent SAS. It can be seen that 55 of the 80 sera had more capacity to bind I*RW than did the nonallergic or ragweed-sensitive controls. Of the 25 sera from the immunized group that precipitated less than 15 per cent of I*RW, 8 were obtained from patients who had received a single injection of only 193 protein nitrogen units (PNU) ragweed pollen extract in adjuvant. The failure of these sera to precipitate additional I*RW may be a reflection of the small antigenic stimulation. On the other hand, at least 10 of the 25 sera with less than 15 per cent binding came from patients who had received aqueous ragweed pollen extract for more than a year followed by 500 to 5,000 PNU of ragweed pollen extract in adjuvant. It seems likely that this latter group of patients were unable to produce antibody against the I*RW antigens as measured by this method. Analogous unresponsiveness to other antigens in amounts which stimulate detectable antibody production in most subjects has been previously described in humans¹³ and in experimental animals.¹⁴ A detailed presentation of this and other data as related to clinical observations will be the subject of a subsequent report.¹⁵

8. Reproducibility of Data Obtained Using I*RW Prepared on Separate Occasions.

During the course of these experiments a pair of selected control sera were repeatedly studied in order to determine the reproducibility of data obtained using different preparations of I*RW. Table VI compares 3 different preparations of I*RW on 8 separate occasions with respect to the per cent precipitated by the reference standard serum and by a single sample of serum from a ragweed-sensitive immunized patient. It can be seen that the antiragweed serum bound an average of 39.0 ± 2.3 per cent with a maximum spread of 8.2 per cent; under the same conditions the reference standard serum bound only 11.0 ± 1.8 per cent with a maximum spread in the data of 6.4 per cent. Of greater importance than the per cent I*RW precipitated by a given antiserum, however, is the difference between the per cent I*RW precipitated by the antiserum and the per cent precipitated by the reference standard serum when determined concomitantly with the same preparation of I*RW. This difference represents the *specific binding* of I*RW by the antiserum which can be attributed to the presence of antibody. It can be seen in Table VI that the *specific binding* of this patient's antiserum, studied on 8 occasions, averaged 28.0 ± 1.8 per cent. Thus, employing 3 different preparations on 8 occasions of I*RW, the method appears to be reproducible to ± 1.8 parts in 28.0 or a standard deviation of approximately 6.5 per cent of the specific binding of the antiserum. Additional

data collected over a period of 9 months are also available for the Reference Standard serum alone. With 9 preparations of I*RW and on 20 separate occasions, the RSS precipitated 11.0 ± 1.5 per cent with a range of 8.7 to 13.9 per cent. In addition, the data indicate that the ragweed pollen extract antigens labeled with I^{131} , and the human antiragweed antiserum used for testing the antigens, remained stable at -20° C. for 3 months. The stability of the data from the repeated tests of the Reference Standard serum suggests that the extract may remain stable for a longer period of time.

TABLE VI. REPRODUCIBILITY OF SPECIFIC BINDING OBTAINED USING I*RW PREPARED ON DIFFERENT OCCASIONS

PREPARATION	% OF 0.02 γ I*RW N/ML. PRECIPITATED		
	RAGWEED POLLEN IMMUNIZED SERUM (43D)	REFERENCE STANDARD SERUM (RSS)	SPECIFIC BINDING (43D-RSS)
A - 1	38.3	11.4	26.9
B - 1	35.3	7.4	27.9
B - 2	40.9	9.3	31.6
B - 3	39.3	10.4	28.9
C - 1	39.0	12.0	27.0
C - 2	38.0	11.8	26.2
C - 3	43.5	13.8	29.7
C - 4	37.8	11.9	25.9
Average	$39.0 \pm 2.3^*$	$11.0 \pm 1.8^*$	$28.0 \pm 1.8^*$

*1 Standard deviation.

9. Lack of Effect of Heat-Labile Serum Components Upon the Specific Binding I*RW by Antisera.

To learn whether heat would effect the I*RW-antibody interaction as measured by this method, samples of sera from nonallergic, untreated ragweed-sensitive, and immunized ragweed-sensitive patients were divided into two aliquots: one aliquot of each was kept as a control and one was heated in a water-bath at 56° C. for 2 hours. It was found that heating neither enhanced nor inhibited the precipitation of I*RW by any of the sera from the three groups of patients tested.

10. Comparison of I*RW-Ammonium Sulfate and Hemagglutination Techniques.

Representative sera were selected and studied with both the I*RW-ammonium sulfate and the bisdiazotized benzidine (BDB) hemagglutination techniques in order to learn if the two methods were measuring the same antibody response to ragweed pollen extract. The I*RW binding as measured with 40 per cent SAS was determined on 6 sera and these sera sent as unknown samples to the laboratory of Dr. Alce H. Schon who performed the BDB hemagglutination determinations. The ragweed pollen extract used for the hemagglutination test was similar to the extract used for the preparation of I*RW except for the fractionation with 50 per cent SAS prior to I^{131} labeling. Three of the sera were sent as duplicate samples and it can be seen in Table VII that the paired data obtained from the 3 sets of duplicates did not vary more than 1 tube.

It can also be seen in Table VII that (a) neither method was able to distinguish between the sera of two untreated ragweed-sensitive individuals with

positive intradermal tests (16A and 33A) and the Reference Standard serum (RSS), and (b) both methods could detect increased antibody activity in the serum from a single individual (16B compared to 16A) 1 month after a single injection of 500 PNU of ragweed pollen extract in adjuvant.

TABLE VII. COMPARISON OF AMMONIUM SULFATE AND HEMAGGLUTINATION TECHNIQUES

HUMAN SERA		40% SAS % OF 0.02 γ I*RW N/ML. PRECIPITATED	BDB HEMAGGLUTINATION RECIPROCAL OF DILUTION TITER
Reference Standard	RSS #1	10.8	80
	RSS #2		160
Ragweed-sensitive	#16A	13.1	40
	#33A	9.5	10
Treated ragweed-sensitive	16B	29.7	160
	7B-#1	20.2	160
	7B-#2		160
	18B-#1	30.0	1,280
	18B-#2		2,560

It is not of particular significance that all 3 post-immunization sera (16B, 7B, and 18B) had high binding by the SAS method inasmuch as they were selected on that basis for evaluation by the BDB method. It is of significance, however, that the two methods do not appear to have measured the same antibody response because (a) the BDB method was unable to distinguish between the nonallergic reference serum (RSS) and 2 of the 3 post-immunization sera (16B and 7B), and (b) the SAS method was unable to differentiate between the two post-immunization sera with the highest binding capacity (16B and 18B), whereas the BDB method indicated a high titer for one (18B) and a low titer for the other (16B).

Thus, it appears possible to further characterize a human antiserum against ragweed pollen by considering data obtained by these two techniques.

DISCUSSION

The use of ammonium sulfate to precipitate soluble complexes formed from the interaction of antibody and some of the I¹³¹ labeled antigens in ragweed pollen extract offers a sensitive and quantitative method for detecting circulating antibody produced by humans and rabbits after the parenteral administration of ragweed pollen extract. That the technique measures antibody has been demonstrated by the specific inhibition of I*RW binding following the addition of unlabeled ragweed pollen antigens (Table IV), by the failure of unrelated antigens to inhibit this interaction (Table IV), and by the failure of antisera against unrelated antigens to bind I*RW (Table III). The antigens and antisera used to demonstrate the specificity of this method were purposely selected so that the possibility of shared or similar antigenic groups was remote. However, as mentioned in association with the data presented in Table V, ragweed pollen extract probably contains antigenic groups similar to antigens derived from other plant sources, such as digitalis leaf, grasses, and trees.¹²

It is apparent from Table V that the method of measuring anti-RW, as it is described in the present report, does not detect the amounts of skin-sensitizing antibody present in the sera of nonimmunized ragweed-sensitive individuals. There are at least three different interpretations as to why the method failed to differentiate between sera of nonallergic individuals and those of nonimmunized ragweed-sensitive individuals: (1) There is evidence to indicate that skin sensitizing antibody-pollen interaction requires the presence of cells¹⁶ or particulate materials¹⁷ before it can be demonstrated *in vitro*. For example, Gordon and his associates⁴ were able to show specific *in vitro* absorption of skin-sensitizing antibody to ragweed with red blood cells coated with ragweed by the BDB method. This observation suggests that it should be possible to detect skin-sensitizing antibody with a red blood cell-ragweed pollen conjugate. However, in a careful statistical study which employed both the BDB and the tanned cell hemagglutination techniques, Arbesman and co-workers¹⁸ were unable to distinguish for the most part between the sera of normal and nonimmunized ragweed-sensitive individuals. (2) Although the I¹³¹ labeled ragweed pollen extract was capable of eliciting a positive intradermal wheal-and-flare reaction when tested on a ragweed-sensitive individual, it is possible that the I¹³¹ label was not affixed to those components of ragweed pollen extract which elicited the positive test. This problem might be resolved by the use of purified fractions of ragweed pollen extract labeled by means of growing ragweed plants in an atmosphere containing C¹⁴.¹⁹ (3) It is entirely possible that there is too small an amount of skin-sensitizing antibody present in the sera of nonimmunized ragweed-sensitive individuals to be detected by this method. Some of the increased binding observed in sera from immunized ragweed-sensitive individuals may be due to an increased amount of skin-sensitizing antibody shown to be present in such sera by Sherman.²⁰ This consideration would be extremely important if a ragweed pollen skin-sensitizing antibody interaction involved easily dissociated or non-avid bonds. Under these conditions small amounts of antibody are difficult to detect.⁵

There is no evidence in the present data to favor one or any of the three alternatives just presented as possible reasons for not detecting increased I*RW binding by the sera of the nonimmunized ragweed-sensitive group. The failure to alter I*RW binding by heating sera to 56° C. does not help to answer the question, despite the fact that human sera containing skin-sensitizing antibody loses its capacity to evoke a positive Prausnitz-Küstner (P-K) reaction under these conditions. It is not known whether 56° C. destroys the capacity of skin-sensitizing antibody to interact with ragweed pollen antigens or whether heat changes some other quality of the antibody molecule so that a positive P-K reaction can no longer be elicited. For example, heating normal skin *in vitro* for half an hour at 60° C. destroys that attribute of skin which is capable of binding skin-sensitizing antibody.¹⁶ Similarly, it is possible that heat could alter the capacity of skin-sensitizing antibody to fix to skin without altering the antigen-binding capacity *per se*. Because of these considerations and until more is known about how skin-sensitizing antibody interacts with skin and

antigen to cause a positive P-K reaction, it does not seem justified to use the thermostability of an *in vitro* antibody test as a criterion to decide whether or not the method in question can detect skin-sensitizing antibody.

The ragweed pollen extract used in the present extracts is a complex antigenic mixture which, in a similar form, has been used by Gordon⁴ and Arbesman¹⁸ and their co-workers for the hemagglutination method. Although the I*RW extract was further fractionated with ammonium sulfate, it undoubtedly remains a complex mixture of antigens. More recently, methods to prepare highly purified fractions of ragweed pollen are becoming available.^{21, 22, 23} It will be of interest to repeat the present studies using those fractions which can be labeled with I¹³¹ and which have the necessary solubility characteristics in ammonium sulfate. In addition, unlabeled purified fractions will be of assistance in elucidating the antigenic mosaic of the relatively crude I*RW used in the present study. This can be done by determining the inhibition activity per microgram of purified material when employed in the inhibition test illustrated in Table IV.

SUMMARY

1. Ragweed pollen extract was fractionated with 50 per cent saturated ammonium sulfate (SAS) and the supernatant labeled with I¹³¹.

2. When 0.02 γ N of this heterogenous mixture (I*RW) was incubated with normal human or rabbit sera, approximately 10 per cent was nonspecifically precipitated with the globulins after the addition of 40 per cent SAS.

3. After incubation with sera from humans or rabbits immunized with ragweed pollen extract as much as 40 to 60 per cent of the I*RW was precipitated in 40 per cent SAS.

4. Lesser degrees of increased precipitation of I*RW were demonstrated with as little as 0.5 ml. of a 1:100 ragweed-injected human antiserum or 0.5 ml. of a 1:2000 ragweed-immunized rabbit antiserum.

5. That the increased I*RW precipitated by immune sera after the addition of 40 per cent SAS was due to binding by specific antibody rather than non-specific co-precipitation was evidenced by: (a) rabbit antisera of high titer for bovine albumin, bovine globulin, human globulin, and Type 12 streptococcal M-protein precipitated no more I*RW than did normal rabbit serum; (b) excess unlabeled ragweed pollen antigens completely inhibited the binding of I*RW by human or rabbit anti-ragweed sera; and (c) apparently unrelated antigens did not inhibit the reaction.

6. Determinations of the precipitation of I*RW on different occasions by a pair of selected control sera demonstrated the reproducibility of the method and the stability of the labeled extract.

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