

ORIGINAL ARTICLES

Immunologic and clinical properties of two purified ragweed fractions

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During 1962, forty-five patients with ragweed hay fever received either multiple aqueous injections or a single repository injection of one of two purified ragweed fractions—DWSR or fraction delta. The purified ragweed fractions were immunologically active, inasmuch as they produced measurable changes in the titers of skin-sensitizing, blocking, and hemagglutinating antibodies in the majority of patients so treated. However, there was no statistically significant correlation between relief of symptoms and changes in antibody titers. The degree of clinical improvement was similar to that produced by hyposensitization with whole ragweed extract. The principal clinical advantages of these purified fractions are their stability, convenience of handling, and reliability of standardization.

Several reports have been published recently of immunologic and clinical studies of specific hyposensitization therapy with whole aqueous extracts of ragweed pollen.¹⁻⁶ The present investigation was undertaken as part of a long-term

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program to assess the immunologic and therapeutic properties of two purified protein fractions prepared from the whole aqueous extract of ragweed pollen (WSR). These two fractions are (a) DWSR, the dialyzed residue of WSR, and (b) fraction delta, a fraction prepared by paper-block electrophoresis from DWSR.

The preparation and the chemical and serologic properties of the ragweed fractions used have been fully described elsewhere⁷⁻⁹ and will therefore be summarized only briefly here. DWSR consists mainly of protein (~60 per cent) and is associated with some carbohydrate and pigment materials.⁷ On the basis of weight DWSR is at least ten times more skin active than WSR and contains all the antigenic and allergenic determinants present in WSR.^{8, 9} Fraction delta has a higher protein content (~80 per cent) but also contains some carbohydrate and pigment.⁷ It is at least a hundred times more skin active than WSR and, like DWSR, contains all the antigenic and allergenic determinants present in WSR.^{8, 9}

The principal objective of this investigation was to determine whether these purified ragweed fractions with high skin activity were also capable of inducing an antibody response in human subjects. In addition, it was hoped that a preliminary impression of the clinical effectiveness of these preparations could be gained from the same series of experiments. During 1962, between March and the beginning of the ragweed hay fever season, forty-five patients with typical symptoms of ragweed hay fever and with positive intradermal skin tests to WSR received a course of hyposensitization injections to one of these two fractions, prepared either as an aqueous solution or as an oil emulsion for repository therapy. Titers of skin-sensitizing, blocking, and hemagglutinating antibodies were determined for the sera of these patients before and after treatment. The therapeutic results were recorded and compared to those obtained with whole WSR either in the same group of patients studied during previous pollen seasons or in a comparable group of patients studied during the same pollen season.

MATERIALS AND METHODS

DWSR and fraction delta

DWSR was prepared from short ragweed pollen collected in 1960 and supplied by the National Institute of Allergy and Infectious Diseases through its Committee on Allergen Standardization. The method of preparation described previously⁷ was used with one modification: This pollen was defatted with cold ether prior to aqueous extraction in order to eliminate difficulties in filtrations encountered during later stages of the preparation. Fraction delta was prepared from DWSR exactly according to the previous method.⁷

Preparation of solutions for aqueous injection

Stock solutions of DWSR (1 mg. per milliliter) and of fraction delta (0.5 mg. per milliliter) were prepared in distilled water and sterilized by filtration through Oxoid membranes* into sterile bottles. Aliquots of 1 ml. volumes of each

*Purchased from British Drug Houses, Toronto, Ontario, Canada.

solution were added under sterile conditions to small vials and were lyophilized; the vials were rubber-stoppered, metal-capped, and stored at 5° C. in a vacuum desiccator until required.* Owing to the instability of these ragweed fractions in solution, fresh solutions were always prepared as required by the addition of the appropriate volume of sterile buffered saline.

Preparation of emulsions for repository therapy

Sterile stock solutions of DWSR (2 mg. per milliliter) and fraction delta (1 mg. per milliliter) were diluted suitably according to the dose to be injected. Six milliliters of each solution was mixed with 4 ml. of a solution of Arlacel in Drakeol (10 per cent v/v) in a syringe attached to a Virtis "23" homogenizer. The mixture was homogenized at 23,000 r.p.m. for 3 minutes on three successive occasions and then transferred through a double-hub needle to 1 ml. syringes for injection. Sterile precautions were used throughout this procedure. Each patient received 1 ml. of the appropriate emulsion intramuscularly.

Classification of patients

The patients were divided into two main groups: twenty-nine patients who had received injections with WSR in previous years (hereafter called previously treated patients), and sixteen patients who had never before received hyposensitization treatment with ragweed pollen extracts (hereafter called previously nontreated patients). Ten of the previously treated patients received DWSR, and another eleven treated patients received fraction delta in a single repository injection. The remaining eight previously treated patients and all the previously nontreated patients were immunized according to standard hyposensitization schedules with multiple injections of aqueous solutions of either DWSR or fraction delta. Of these, three previously treated patients and seven previously nontreated patients received multiple aqueous injections of DWSR, and five previously treated patients and nine previously nontreated patients received multiple aqueous injections of fraction delta. For a comparison of the clinical efficacy of DWSR and fraction delta in previously nontreated patients, six were given multiple aqueous injections of WSR.

Dosage and treatment

The dosage schedule for each patient was determined by (a) direct skin testing, (b) severity of clinical symptoms, and (c) previous experience with hyposensitization therapy, when applicable. The total amounts given throughout the treatment varied between the following limits: by multiple aqueous injections, 0.1 to 2.5 mg. of DWSR and 0.05 to 1.25 mg. of fraction delta; by repository injections, 0.1 to 1.0 mg. of DWSR and 0.05 to 0.5 mg. of fraction delta. It was found that 1 μ g of DWSR or 0.5 μ g of fraction delta had approximately the same skin activity as 10 PNU of WSR. Treatment with multiple aqueous injections was started in March and continued until the beginning of the ragweed season in

*We wish to thank Ayerst, McKenna, and Harrison Ltd., Montreal, Quebec, Canada, for lyophilizing these materials under sterile conditions and for supplying the vials.

Table I. *Typical dosage schedules for treatment with fraction delta* by multiple aqueous injections*

<i>Previously nontreated patient</i> (μ g)	<i>Previously treated patient</i> (μ g)
0.125	1.0
0.25	2.0
0.50	3.5
0.75	5.0
1.00	10.0
1.75	15.0
2.50	25.0
3.50	35.0
5.00	50.0
7.50	75.0
10.0	100.0
15.0	150.0
20.0	200.0
30.0	250.0
40.0	300.0
50.0	400.0

*The corresponding doses of DWSR were double.

August, for a total of about 15 injections. Treatment was not maintained during the season. A typical hyposensitization schedule for the purified ragweed fractions is illustrated in Table I. Repository treatment was administered by one injection per patient during the latter half of June.

Collection of sera

Samples of sera were collected on four occasions: (a) before treatment in March (pretreatment), (b) in May, (c) before the ragweed season in August, and (d) after the ragweed season in October (postseasonal). Unfortunately, samples of sera were not available from all of the patients in May. The sera were Scitz-filtered and stored in the cold until required.

Skin-sensitizing antibody titers

Passive transfer (P-K) tests were performed on the backs of normal non-allergic volunteers. The P-K titers of the sera were determined in the manner previously described⁷ with WSR used as the challenging antigen and were expressed as the reciprocal of the highest dilution which still gave a positive reaction. Control sites on the backs of each volunteer were sensitized with dilutions of a serum of known P-K titer so that the titers of all the sera in the several experiments could be compared directly.

Blocking antibody titers

The test for blocking antibody was performed as described previously¹⁰ with WSR as the neutralizing and challenging antigen. The same standard reaginic serum from a nontreated allergic individual was used in all experiments. Each test was performed in duplicate at the same time on the backs of two normal volunteers. The amount of blocking antibody was expressed in terms of the amount of WSR (in PNU) inactivated by 1 ml. of serum.

Hemagglutination titers

Hemagglutination titers were determined by the standard BDB technique described previously¹¹ and by a micro adaptation of it, with the Microtit set devised by Takátsy.¹² In a blind study* with fifty sera from ragweed-sensitive individuals, both methods were shown to give identical titers within a factor of ± 2 .

The microhemagglutination test was performed in translucent plastic plates, 8 by 68 by 130 mm., which contained 6 rows of 12 wells, each 6 mm. in diameter. Each well had a funnel-shaped base and a volume of 0.15 ml. The diluent was delivered to the wells by means of a rust-free calibrated metal tube enclosed in plastic and attached to a pipette. Two drops of the diluent (0.05 ml.) were placed in each well. Twofold serial dilutions of the sera, from which nonspecific agglutinins had previously been removed by absorption with nonsensitized cells, were made with a spiral loop by successively transferring the contents of the loop from one well into the next. A volume of 0.025 ml. of a 1 per cent suspension of sensitized cells was added to each well. The plates were gently shaken to disperse the cells. The reaction proceeded at room temperature and was read after a minimum of 1 hour, usually after 4 to 6 hours. Since the volume ratio of sensitized cells to serum differs in the macro- and micromethods, different conditions for optimum sensitivity were established for the micromethod and found to be as follows: A saline solution of WSR (3 mg. in 1 ml.), 0.1 ml. of a 50 per cent suspension of red blood cells, and 0.5 ml. of fifteenfold diluted BDB solution¹¹ were incubated at room temperature for 15 minutes. The sensitized cells were centrifuged, washed, and resuspended in diluent to give a 1 per cent suspension, as described previously.¹¹

Evaluation of the clinical results of treatment

Preliminary clinical studies were undertaken in order to compare the therapeutic effect of the purified ragweed fractions with that of whole ragweed extract. During the first week in October, each patient was interviewed and asked to compare the severity of symptoms during the 1962 season with those experienced during the 1961 season. The amount of antihistamine medication (chlorpheniramine maleate) used by each patient during the ragweed season was determined (a) by direct questioning and (b) by subtracting the amount of medication still in the patient's possession from the amounts which had been supplied during the season. Since this was a group of indigent patients who received drugs at token cost from the government-subsidized hospital pharmacy, there was little motivation for them to obtain medication from outside sources.

In the previously treated group of patients, the amount of medication used during the 1962 pollen season was compared with the average amount used by the same patient during the previous 1 to 4 seasons. A scoring system was devised in which the patient's subjective evaluation of symptoms and the comparative quantity of antihistaminics used in 1962 were given equal weight. On this basis, the treatment results with DWSR and fraction delta were classified as "better," "the same," or "worse" than those obtained in previous seasons with whole WSR.

*We wish to thank Mrs. A. Reiner and Dr. L. Gyenes for these results.

In the previously nontreated group of patients, no data on symptoms or medication were available from previous years. Therefore, the treatment results with purified ragweed fractions were based only on the patients' reports of whether they were "better," "the same," or "worse" than during the 1961 ragweed season, when they did not receive injection therapy. For comparison, a matched group of previously nontreated patients was given hyposensitization therapy with whole WSR and evaluated in the same way.

The 1962 ragweed pollen season was a comparatively heavy one in the Montreal area. Total ragweed pollen counts recorded at the Montreal General Hospital pollen station for 1962 and preceding years are as follows: 1962—2,232; 1961—590; 1960—880; 1959—1,564; 1958—1,077.

RESULTS

Skin-sensitizing antibody titers

No general pattern in the change of the titers of skin-sensitizing antibodies was observed in the sera of the twenty-four patients treated by aqueous injections of DWSR or of fraction delta, the actual titers varying from less than 100 to more than 1,200. The level of antibody (a) of four patients remained unchanged, (b) of ten patients increased and remained above the pretreatment level, (c) of six patients decreased, and (d) of four patients varied by a factor ± 2 but returned to the pretreatment level. The P-K titers of the sera of patients treated by repository therapy were found practically unchanged at the end of the season, the titers being less than or equal to 100.

Blocking antibody titers

The blocking antibody titers of fourteen samples of the pretreatment and of the corresponding postseasonal sera were determined and are recorded in

Table II. *Blocking antibody titers of pretreatment and postseasonal sera*

Patient	Class*	Treated with ragweed fraction	Titers (PNU blocked by 1 ml. of serum)		Clinical result
			Pretreatment	Postseasonal	
Aqueous					
C. C.	Non-T	DWSR	0	320	Better
E. B.	T	DWSR	0	80	Better
E. R.	T	DWSR	0	80	Worse
D. M.	Non-T	DELTA	0	640	Better
N. H.	Non-T	DELTA	0	160	Better
R. K.	Non-T	DELTA	0	640	Better
M. C.	T	DELTA	160	> 640	Better
Repository					
A. M.	T	DWSR	80	320	Better
A. D.	T	DWSR	320	640	Same
L. H.	T	DWSR	320	320	Same
E. S.	T	DWSR	640	> 640	Worse
R. S.	T	DELTA	80	160	Worse
M. C.	T	DELTA	0	160	Worse
J. B.	T	DELTA	160	320	Worse

*Non-T = previously non-treated; T = previously treated.

Table III. Examples of the variation of BDB-hemagglutination titers during and after treatment

Patient	Class*	Treated with ragweed fraction	Serum sample†				Clinical result
			Pre-T	May	August	Post-S	
Aqueous							
L. W.	Non-T	DWSR	20	20	80	160	Better
S. G.	Non-T	DWSR	60	240	1,280	1,280	Better
E. B.	T	DWSR	160	1,280	640	1,280	Better
N. H.	Non-T	DELTA	< 10	160	640	1,280	Better
A. R.	T	DWSR	480	1,280	2,560	2,560	Worse
I. M.	T	DELTA	60	480	2,560	2,560	Worse
Repository							
M. R.	T	DWSR	160			1,280	Better
F. P.	T	DELTA	1,280			2,560	Worse
M. C.	T	DELTA	40			640	Worse

*Non-T = previously non-treated; T = previously treated.

†Pre-T = pretreatment; Post-S = postseasonal.

Table II. The titers of all sera increased, except that from one patient. The antibody titers of a further sixteen samples of the postseasonal sera were determined. Of the entire thirty patients whose postseasonal sera titers were measured, seventeen had had a better season, eleven a worse season, and two were judged to have fared the same. Twelve of the "better" and four of the "worse" patients had titers of 320 or higher, whereas five of the "better" patients and seven of the "worse" patients had titers of 160 or less.

Hemagglutination titers

The BDB hemagglutination titers of the sera of all of the patients who had been treated by aqueous injections were observed to increase during treatment with either DWSR or fraction delta. It was possible to examine both the pretreatment and postseasonal sera of only seventeen of the twenty-one patients who received repository therapy, and, of these, all but two sera had an increased titer after treatment. Some examples of the variation of hemagglutination titers are presented in Table III.

Clinical evaluation

The clinical results of treatment are summarized in Tables IV and V. It will be noted that a somewhat greater degree of clinical improvement over the previous season was obtained in the previously nontreated group of patients than in the previously treated group. When the data for the previously treated patients are further analyzed, it is apparent that the clinical effect produced by hyposensitization injections with DWSR or fraction delta was comparable to that produced in previous seasons by whole WSR (Table IV). No significant difference in therapeutic results was observed between DWSR and fraction delta or between the aqueous and repository methods of administration in this group of patients.

In a small number of previously nontreated patients, the degree of clinical improvement over the previous season obtained with both DWSR and fraction

Table IV. *Clinical effect of DWSR and fraction delta in patients previously treated with whole WSR*

<i>Antigen</i>	<i>Vehicle</i>	<i>Number of patients</i>		
		<i>Better</i>	<i>Same</i>	<i>Worse</i>
DWSR	Aqueous	1	0	2
DWSR	Repository	4	2	4
Fraction delta	Aqueous	3	0	2
Fraction delta	Repository	4	2	5

Table V. *Clinical effect of DWSR, fraction delta, and whole WSR in previously nontreated patients*

<i>Antigen</i>	<i>Vehicle</i>	<i>Number of patients</i>		
		<i>Better</i>	<i>Same</i>	<i>Worse</i>
DWSR	Aqueous	7	0	0
Fraction delta	Aqueous	8	0	1
WSR	Aqueous	3	2	1

delta appeared to be superior to that obtained with whole WSR (Table V). The frequency and severity of local swelling and systemic reactions which occurred with the purified ragweed fractions was approximately the same as with whole ragweed extract.

DISCUSSION

The data presented here form the first part of a long-term program of evaluation of DWSR and fraction delta over several ragweed hay fever seasons. Therefore, any conclusions which can be drawn at this time must necessarily be considered provisional.

However, it is clear from the data obtained in this investigation that DWSR and fraction delta are immunologically active when administered by injection to human subjects. Furthermore, preliminary clinical evaluation would suggest that hyposensitization treatment with these purified ragweed fractions produces approximately the same degree of clinical improvement in ragweed hay fever as does treatment with whole ragweed extract. The therapeutic effectiveness of treatment with whole ragweed extract has already been demonstrated by other workers.⁵ Contrary to preliminary expectations, the incidence of local irritation or systemic reactions with injection therapy was not appreciably lowered by purifying the ragweed extract.

Nevertheless, these purified ragweed preparations have several practical advantages in clinical practice. For example, the deterioration of allergenic preparations in aqueous solution on long storage even in the cold is well known. The purified ragweed fractions, on the other hand, were stored in the dry state without any detectable deterioration of specific activity. For use in the clinic, it was found convenient to supply sterile serum vials containing 1 mg. of DWSR and 0.5 mg. of fraction delta as lyophilized powders. Fresh solutions of desired

concentrations could then be prepared in the allergy clinic by the addition of sterile buffered saline through the rubber cap. In this way, solutions standardized in micrograms of a dry powdered material could easily be prepared, and variations in potency due to deterioration or to arbitrary unitage systems were avoided without extra manipulation.

No correlation could be discovered between the dosage administered and either the degree of clinical improvement or the titers of skin-sensitizing, blocking, and hemagglutinating antibodies, thus confirming the experience of other workers.^{3, 13} Furthermore, there was no apparent relation between the change in the level of skin-sensitizing antibodies and either clinical relief or the ragweed fraction used in treatment. In a relatively high proportion of cases (ten out of twenty-four), the P-K titers increased during treatment and decreased only slightly, if at all, during the season; similar changes had been previously observed in studies in which the whole ragweed pollen extract had been used for immunization.^{1, 14}

Some correlation was found between the blocking antibody titers and the extent of relief obtained. Twelve of the seventeen patients who had had a better season had postseasonal titers of 320 or higher, while seven of the eleven patients who had had a worse season had titers lower than 320. The relative increase in the blocking titers of fourteen patients was measured and the results are summarized in Table II. Although DWSR and fraction delta had been used in treatment, the antibody titers in all cases were measured in terms of the amount of WSR neutralized. This procedure was adopted since it had been previously shown that DWSR and fraction delta possess all the antigenic and allergenic determinants of WSR.^{8, 9} Nevertheless, it ought to be stressed that it does not necessarily follow that the blocking antibodies thus measured were directed against all the determinants of ragweed pollen. In the present study, the blocking antibodies produced on treatment with WSR were not measured. However, it can be stated that the rate of production of blocking antibodies, on treatment with DWSR or fraction delta by multiple injections, was more marked than has been observed in this laboratory with WSR by the same method.

The BDB hemagglutination titers of all but two sera increased after treatment with either of the two ragweed fractions. The postseasonal sera of the patients receiving aqueous injections were placed arbitrarily in three groups, namely, those which had low, medium, or high titers (see Table VI). It will be noticed that patients who had a "better" season were found in all groups, but the highest proportion of their sera had high hemagglutination titers. In all three categories, no particular significance could be attached to the ragweed fraction used in treatment or to whether the patient had previously received treatment or not. It is worth mentioning that all the patients with medium or high titers who had had a "worse" season also had low blocking antibody titers of 80 or less.

The weight of available experimental evidence¹⁵ points to the fact that blocking antibodies are normal immune divalent antibodies and that, although their contribution to the hemagglutination titers is large, they are not the only factor measured in this serologic test. The participation of skin-sensitizing antibodies in the hemagglutination reaction, as well as of antibodies directed to ragweed anti-

Table VI. *Distribution of hemagglutination titers of the postseasonal sera of patients treated by aqueous injections of DWSR or fraction delta*

Fraction	Class*	Low titers 0—160			Medium titers 320—640			High titers 1,280—5,120		
		B	S	W	B	S	W	B	S	W
DWSR	Non-T	2	0	0	2	0	0	3	0	0
	T	0	0	0	0	0	1	1	0	1
Delta	Non-T	2	0	0	1	0	1	5	0	0
	T	0	0	0	1	0	1	2	0	1
Total number of patients		4	0	0	4	0	3	11	0	2

*Non-T = previously non treated; T = previously treated; B, S, and W = better, same, and worse.

gens other than those responsible for the production of skin-sensitizing and blocking antibodies, cannot yet be ruled out. It is not surprising, therefore, that there is at best only partial correlation between the extent of clinical relief and hemagglutinating or blocking antibody titers of whole serum. As has been shown,¹⁰ the bulk of the hemagglutinins is associated with a serum fraction (obtained by chromatography on DEAE-cellulose) containing mainly blocking antibodies, and the largest portion of skin-sensitizing antibodies is found in a fraction possessing only a small hemagglutination titer. Moreover, the hemagglutinins of each of these two serum fractions were shown to comprise a heterogeneous population of antibody molecules and to vary from serum to serum. It is suggested, in view of this multiplicity of antibodies, that a closer correlation might be found between clinical relief and the hemagglutination titers of distinct serum fractions rather than the titers of whole sera.

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