PRECIPITATING ANTIBODIES OF HUMAN ANTISERA ELICITED BY A PURIFIED DWARF RAGWEED FRACTION

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GENERALLY, it has not been possible to demonstrate the presence of precipitins in whole human antisera to ragweed extracts.[1-3] Two reasons have been introduced[4, 5] to explain this situation: (1) ragweed extracts produce an incomplete or univalent antibody in the human which fails to precipitate ragweed antigen, (2) the antibodies are multivalent, but due to their low concentration they have evaded detection by conventional precipitin techniques. However, Follensby[6] has observed precipitin bands during gel diffusion studies with sera of treated and untreated ragweed-sensitive patients. Recently, Feinberg and Cline[7] have devised a method to demonstrate precipitins in the pseudoglobulin fraction of sera of atopic and nonatopic subjects who were treated with ragweed extracts. Subsequently, Perelmutter and his associates[8, 9] and Friedman and Criep[10] have fractionated human ragweed antisera on a diethylaminoethyl-cellulose column and, after concentration of one fraction, have observed a precipitin reaction. Multiple precipitating antigen-antibody systems in human anti-ragweed sera were demonstrated by the micro-Ouchterlony method.[8]

The present investigation describes the microimmunoelectrophoretic demonstration of, and possible factors responsible for, precipitins in unfractionated and unconcentrated antisera of 2 ragweed-sensitive patients who had been treated with a purified dwarf ragweed fraction, Pool C. The whole antisera were further characterized by measuring the hemagglutination titers by means of the tanned cell technique.

METHODS AND MATERIALS

Preparation and Properties of Dwarf Ragweed Allergens.—Pool C was prepared by the method of Goldfarb and Callaghan.[11, 12*] Pool C was derived from DMLL (a lyophilized aqueous extract of defatted dwarf pollen which had been leached previously with methanol) and DI (a water-soluble fraction derived

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*Pool C and precursor samples obtained through the courtesy of Dr. A. R. Goldfarb (Sinai Hospital of Detroit, Michigan) and those prepared by the investigators appeared to be identical with the use of microimmunoelectrophoresis in conjunction with several rabbit anti-ragweed sera.

from DMLL by repeated extraction and precipitation in 90 per cent methanol). Fraction DI was chromatographed on a diethylaminoethyl-cellulose column and eluted with buffered saline at pH 7.4 to produce Pool C. Pool C was subfractionated by adding ammonium sulfate to obtain 60 per cent saturation. The precipitate (Pool C, 0-60), hereafter termed Pool C, was used for hyposensitization.

Pool C possessed greater skin reactivity than precursor fractions.[12] Disc electrophoresis has revealed six protein components.[13] Microimmunoelectrophoresis, using Pool C against a rabbit anti-ragweed antisera, has revealed at least six antigens.[13]

Method of Hyposensitization.—Patients J and S were classified as ragweed-sensitive on the basis of clinical history and positive intradermal responses to whole ragweed extract (1:1000), NIH #508 (a nondialyzable residue of a whole dwarf ragweed extract),* and Pool C. The patients were treated with multiple subcutaneous injections of Pool C in both aqueous and sodium alginate-calcium gluconate adjuvant†[14, 15] systems. All solutions were freshly prepared to minimize possible degradation of the antigens. The 2 subjects (J and S) in whom precipitins were induced were members of a group of 13 ragweed-sensitive subjects who were hyposensitized with Pool C. Twelve subjects, not sensitive to ragweed, were also treated with Pool C and utilized as controls. The results of this study will appear elsewhere.[16]

Hemagglutination.—Antisera were collected and stored at −8° C. The indirect tanned cell hemagglutination titer of all antisera was determined by the method of Boyden,[17] as modified by Arbesman.[18] A "standard coating system" composed of 2 mg. per cent of Pool C in a mixture of equal parts of pH 6.4 buffered saline and pH 7.2 buffered saline was employed to sensitize the tanned red blood cells. The tanned cell hemagglutination titers were obtained by determining the reciprocal of the highest dilution of antisera which showed a positive hemagglutination reaction. Serial twofold dilutions were used, beginning with a dilution of 1:5. A hemagglutination titer reported as zero indicated the titer was less than 1:5.

Microimmunoelectrophoresis.—Microimmunoelectrophoretic studies were started after it was observed that several ragweed-sensitive subjects developed relatively high hemagglutination titers during the early stage of the hyposensitization study. Subsequently, all antisera were subjected to routine microimmunoelectrophoretic analyses. The Shandon Universal electrophoresis and constant voltage units,† Universal agar cutter,‡ and microimmunoelectrophoretic techniques of Hirschfeld[19] were used. Electromigration and diffusion processes were performed at 4° C. to retard possible degradation of the ragweed preparations. Electromigration of Pool C, DMLL, and NIH #508 (10 to 20 mg./ml. buffer) was conducted in 7 per cent Ionagar No. 2 gel† in Veronal-sodium

*Obtained through the courtesy of Dr. R. Wodehouse and C. Gussoni, Lederle Laboratories, Pearl River, New York. This antiserum was described as a pool (No. 90) of bleedings from 12 rabbits hyperimmunized using Freund's complete adjuvant and short ragweed extract No. 508 from pollen received from the National Institutes of Health. In the present paper, the authors have applied the terms NIH #90 and NIH #508 to represent the rabbit antiserum and homologous ragweed extract, respectively.

†Colab Laboratories, Inc., Box 66, Chicago Heights, Illinois.

‡Buchler Instruments Inc., 1327-16th Street, Fort Lee, N. J.

Veronal-calcium lactate buffer of pH 8.6[20] for 90 minutes at a potential gradient of 7 volts per centimeter. A 10 per cent whole small ragweed extract (WSR), prepared in the manner of Arbesman,[18] was used as reference electrophoretic migrant. Samples of whole antisera (neither concentrated nor diluted) were allowed to diffuse for at least 3 days. Under these conditions, the first precipitins were usually visible during the first day of diffusion. Nonprecipitated material was removed from the gels by extraction for 3 days in 0.8 per cent sodium chloride followed by one day in distilled water. The precipitins were stained with 0.1 per cent amido black 10B in the acetic acid-methanol-water system.[19]

RESULTS

Microimmunoelectrophoresis.—Among the sera of 13 ragweed-sensitive subjects and 12 not sensitive to ragweed who were hyposensitized with Pool C only 2 were found to contain precipitins. Microimmunoelectrophoretic analyses of the whole undiluted antisera of 2 ragweed-sensitive subjects (Fig. 1, Subject J; and Fig. 2, Subject S) revealed the presence of precipitating antibodies

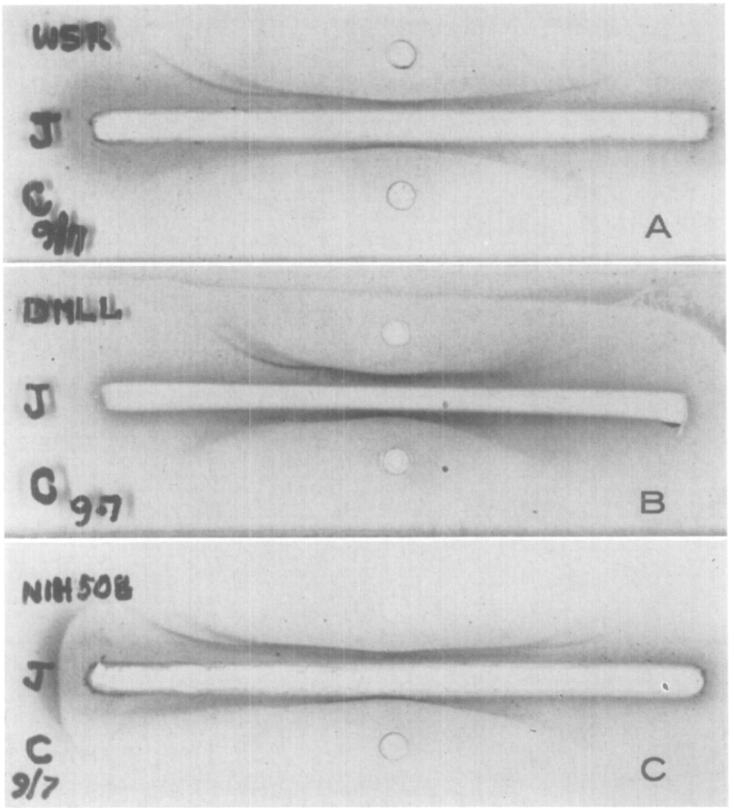

Fig. 1.—Microimmunoelectrophoretic precipitin patterns of whole undiluted Pool C antiserum (in all longitudinal troughs) of ragweed-sensitive Subject J at post-treatment stage. Anode was at left. Pool C (20 mg./ml.) was always placed in the lower circular well of a given slide. The following antigen systems were placed in the upper circular wells as follows: *A*, WSR = a 10 per cent whole dwarf ragweed extract. *B*, DMLL = an aqueous extract of dwarf pollen which had been leached previously with methanol (20 mg./ml.). *C*, NIH #508 = a nondialyzable residue of a whole dwarf ragweed extract (20 mg./ml.).

after reaction with not only the homologous antigen fraction (Pool C) but also with three other ragweed extracts: a whole dwarf ragweed extract (WSR); a nondialyzable residue of a whole dwarf ragweed extract (NIH #508); and a precursor of Pool C (DMLL). Pre-treatment sera of Subjects J and S did not produce precipitin bands under these conditions.

Fig. 3 illustrates the more complex precipitin patterns obtained with the four antigen systems after reaction with the reference (NIH #90) rabbit antiserum. Since minor variations in pattern were encountered, Pool C was always used simultaneously as a reference migrant in the lower wells.

With regard to a particular antigen system, the human precipitin patterns were found to have fewer and less intense bands than the rabbit antiserum. The patterns that were produced by the Pool C antisera of both patients with regard to Pool C were quite similar. Zones of partial identity were also evident. It was apparent that the patterns produced by reaction of human Pool C antisera with Pool C (lower well) and another antigen (e.g., *WSR* in upper well of

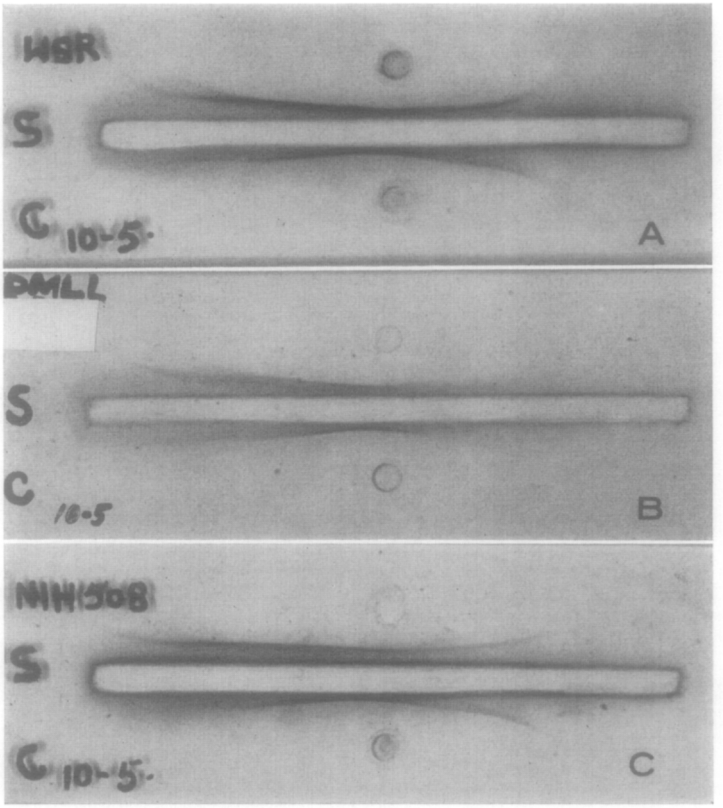

Fig. 2.—Microimmunoelectrophoretic precipitin patterns of whole undiluted Pool C antiserum (in all longitudinal troughs) of ragweed-sensitive Subject S at the post-treatment stage. Anode was at left. Pool C (20 mg./ml.) was always placed in the lower circular well of a given slide. The following antigen systems were placed in the upper circular wells as follows: *A*, WSR = a 10 per cent whole dwarf ragweed extract. *B*, DMLL = an aqueous extract of dwarf pollen which had been leached previously with methanol (20 mg./ml.). *C*, NIH #508 = a nondialyzable residue of a whole dwarf ragweed extract (20 mg./ml.).

Fig. 1, *A*) on a given slide were almost mirror images. Although such a comparison is qualitative at best, it may suggest that Pool C exists as a chemical entity in DMLL, NIH #508, and WSR.

Although only the post-treatment sera are illustrated in Figs. 1 and 2, other antisera of both subjects were analyzed during the course of hyposensitization. Generally, the precipitin patterns were quite similar. A chronological comparison of band intensities produced by a given subject with regard to a specific antigen could be misleading, due to minor variations in electrophoretic technique.

Immunologic Response of Subjects (J and S).—The frequency and dosage of Pool C hyposensitization, tanned cell hemagglutination titers, and time of first observation of precipitins of the 2 subjects are shown in Fig. 4.

The antiserum of Subject J was found to produce a precipitin reaction after the subject had received four Pool C injections (three alginate and one aqueous) during a period of 6 weeks. The cumulative antigen dose at this

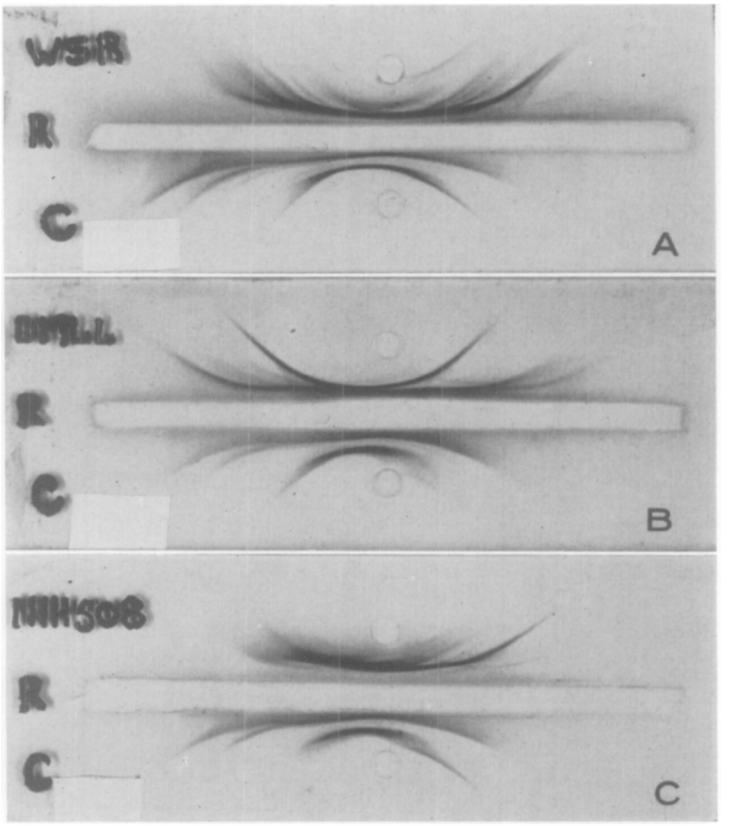

Fig. 3.—Microimmunoelectrophoretic precipitin patterns of NIH #90 rabbit antiserum to a nondialyzable residue of a whole ragweed extract. Undiluted NIH #90 antiserum was placed in all longitudinal troughs. Anode was at left. Pool C (20 mg./ml.) was always placed in the lower circular well of a given slide. The following antigen systems were placed in the upper circular wells as follows: *A*, WSR = a 10 per cent whole dwarf ragweed extract. *B*, DMLL = an aqueous extract of dwarf ragweed pollen which had been leached previously with methanol (20 mg./ml.). *C*, NIH #508 = a nondialyzable residue of a whole dwarf ragweed extract (20 mg./ml.).

point was 380 μg. All antisera obtained at a later date were found to contain precipitins and to produce a similar precipitin pattern. At the end of hyposensitization in September, the subject had received a total cumulative dose of 3,880 μg of Pool C composed of 14 injections (8 alginate, 6 aqueous) during a 28 week interval. The hemagglutination titer (using cells sensitized with Pool C) of the pretreatment serum of Subject J was 1,280. At the time that the precipitin bands were first observed, the hemagglutination titer had risen to 40,960—a thirty-two fold increase. Although the titer decreased to 5,120 in a subsequent antiserum, the precipitins were still demonstrable. The post-treatment titer obtained in September rose again to 40,960.

Precipitins in the antisera of Subject S were first seen at a later date than in Subject J, 23 weeks from start of hyposensitization. At this time, the subject had received 8 injections of Pool C (5 alginate, 3 aqueous) containing a cumulative dose of 1,765 μg. The antiserum had a titer of 20,480, a sixteenfold

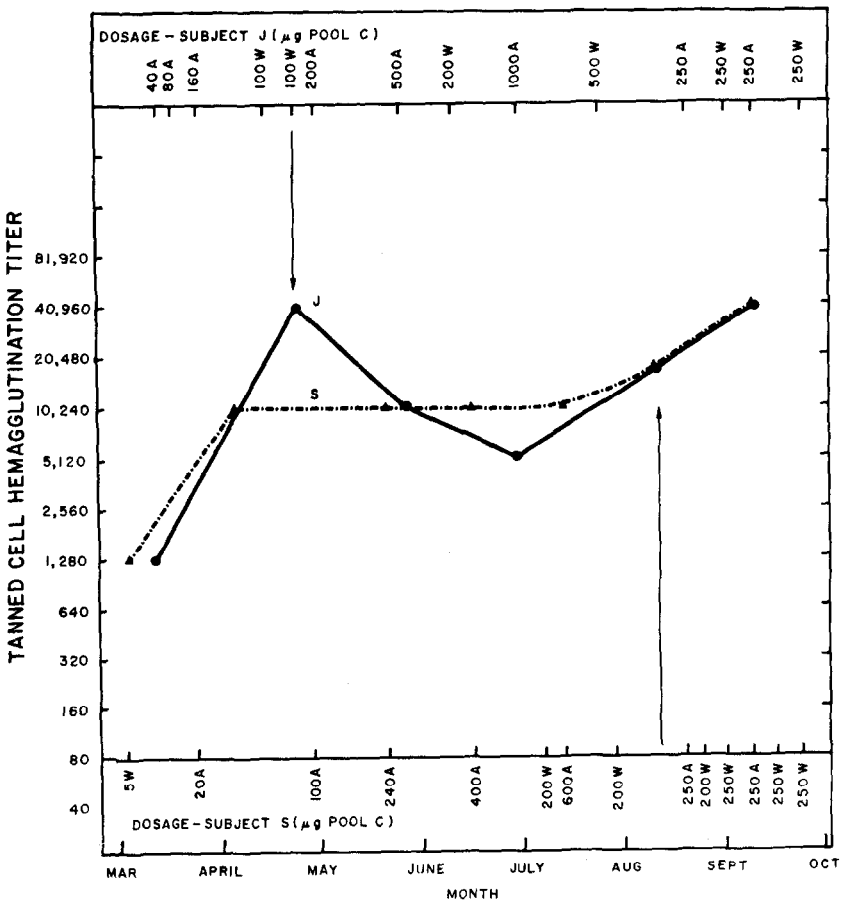

Fig. 4.—The hemagglutination titers of ragweed-sensitive Subjects J and S with the use of tanned cells sensitized with Pool C. Subjects were hyposensitized with alginate (*A*) and aqueous (*W*) solutions containing Pool C. Arrows indicate first observation of precipitin reaction using microimmunoelectrophoresis. Curves ▲----------▲ and ●——————● represent tanned cell hemagglutination titers of Subjects S and J, respectively.

increase over the pretreatment titer. Upon termination of hyposensitization at 27 weeks, the subject had received 14 injections (7 alginate and 7 aqueous) of Pool C with a cumulative dose of 3,215 μg. The initial and final hemagglutination titers of Subject S were 1,280 and 40,960—identical to Subject J. As in the case of Subject J, precipitins once detected were demonstrated in all subsequent antisera.

Effect of Tanned Cell Coating on Pre- and Post-Treatment Hemagglutinating Antibody Levels.—Table I illustrates the variation in titers that was obtained with the use of different antigens to sensitize the tanned cells used to react with the pre- and post-treatment sera of Subjects J and S (unheated sera). Using these "standard coating solutions," cells sensitized with Pool C always produced the highest titers. Pool C treatment of both subjects also produced titer increases when whole dwarf ragweed (WSR) and a nondialyzable residue of whole dwarf ragweed extract (NIH #508) were used to coat the tanned cells. However, in the case of Subject J the increases were not significant.

TABLE I. EFFECT OF TANNED CELL COATING AND HEAT TREATMENT OF SERA ON PRE- AND POST-IMMUNIZATION HEMAGGLUTINATING ANTIBODY LEVELS

	TITERS* OF PRE-IMMUNIZATION SERA TANNED CELL COATING			TITERS OF POST-IMMUNIZATION SERA TANNED CELL COATING		
HUMAN SERA	2 MG. % POOL C	2 MG. % NIH #508	1.5 % WSR‡	2 MG. % POOL C	2 MG. % NIH #508	1.5 % WSR‡
Subject J Unheated	1,280	160	160	40,960	320 NS§	320 NS
Subject J Heated†	1,280	80	80	20,480	160 NS	320 NS
Subject S Unheated	1,280	5	80	40,960	640	640
Subject S Heated†	1,280	10	10	20,480	160	640

*Tanned cell titers are recorded as the reciprocal of the highest dilutions of antisera that produced hemagglutination.

†Sera heated for 4 hr. at 56° C.

‡WSR refers to a whole dwarf ragweed extract obtained by extraction of 10 Gm. pollen with 100 ml. buffered saline, the extract subsequently diluted 1.5 to 10 with buffer prior to coating of tanned cells.

§NS = Not significant. The change in titers between these post-immunization sera and the corresponding pre-immunization sera was not significant. Titers were reproducible within ± 2 tubes (± a fourfold dilution) when the same antiserum was used with different lots of erythrocytes.

Effect of Heat Treatment of Sera on Pre- and Post-Treatment Hemagglutinating Antibody Levels.—Incubation of the pre- and post-treatment sera for 4 hours at 56° C. to inactivate skin-sensitizing antibody did not appreciably alter the hemagglutination titers (Table I). However, the heat treatment diminished the formation of precipitin bands in the post-immunization sera of either patient.

DISCUSSION

It was evident from the present study that several precipitating antibodies were produced in the antiserum of each of 2 patients who were treated with a purified dwarf ragweed preparation—Pool C. Microimmunoelectrophoretic analyses showed the presence of precipitins in the unconcentrated and un-

diluted whole antisera of these patients. Under these conditions, the Pool C antisera reacted not only with Pool C to produce precipitin bands, but also with several whole ragweed extracts. Feinberg and Cline[7] and Perelmutter and his co-workers[8, 9] have been able to demonstrate precipitins in a fraction of human ragweed antisera. The latter investigators have found precipitating antibodies in the sera of untreated ragweed-sensitive patients, treated ragweed-sensitive, and treated patients who were not sensitive to ragweed. In the present investigation, the demonstration of precipitins in the antisera of 2 out of a group of 25 patients (13 ragweed-sensitive patients and 12 not sensitive) appeared to be associated with high hemagglutinating antibody levels. It was found[16] that the post-treatment hemagglutination titers (tanned cells coated with Pool C) of 40,960 for each of the 2 patients were much higher than those of other Pool C-treated, ragweed-sensitive patients (1,280 to 10,240, range; 3,607, mean) or of the Pool C-treated group not ragweed sensitive (20 to 5,120, range; 2,498, mean). The data tended to support the thesis that at least one type of antibody in treated allergic sera is multivalent. This antibody may be demonstrated by indirect hemagglutination and by a method of lesser sensitivity—immunodiffusion in agar gel.

Inasmuch as all patients were given Pool C in the form of both aqueous and sodium alginate systems, it was not possible to ascribe precipitin production solely to the adjuvant. The comparatively high pre-treatment hemagglutination titers (1,280) of Patients J and S may have allowed attainment of high post-treatment levels. Pre-treatment titers of other ragweed-sensitive patients ranged from 0 to 320, with a mean titer of 114. Pre-treatment titers of the patients who were not ragweed sensitive ranged from 0 to 320, with a mean titer of 30. Since both patients had been treated the previous year with whole ragweed extracts, the pre-treatment (with regard to tanned cells coated with Pool C) titers could be expected to be measurable but not necessarily high. Furthermore, it was known that Subject S had a constitutional reaction to whole ragweed extract 2 years prior to the present therapy. The second injection of Pool C (80 μg) into Patient J elicited a constitutional reaction.[16] Possibly the attainment of high post-treatment titers and the development of precipitins may have been facilitated by constitutional reactions in these patients. However, Connell and his group[21] have found that the hemagglutination titers in sera of patients who had constitutional reactions to whole ragweed extracts were less than those of a post-repository group and a group treated with high doses of aqueous ragweed extracts.

The demonstration of precipitating antibodies in antisera that possessed high hemagglutination titers may be without clinical significance. The clinical relief that occurred in Patients J and S was rated as good and excellent, respectively.[16] However, it was quite clear that in these and all other ragweed-sensitive patients who were treated with Pool C, clinical relief was not complete as is also usual with whole ragweed therapy. The small number of patients used for treatment with Pool C precluded a definitive comparison of clinical response obtained with Patients J and S as compared to that obtained with other treated ragweed-sensitive patients.

Heating the antisera for 4 hours at 56° C. diminished, and more often abolished, the formation of precipitin bands. In our hands, this treatment causes a small change in the hemagglutination titer (generally a decrease of one or two tubes). As previously noted, the precipitin bands have developed in antisera having titers of 20,480 (± 2 tubes; cells tanned using standard Pool C system). Heating the antisera may suffice to slightly denature the precipitins, inhibit formation of precipitin bands, and decrease the hemagglutination titer. The experimental error of the hemagglutination technique prevents a more exact evaluation of the precipitin-threshold titer. Certainly the role of reagin was not clear in this process. Multiple precipitating antibody systems to a nondialyzable whole ragweed extract following fractionation of the antisera and concentration of a specific fraction have been demonstrated recently.[8, 9] Since most of the hemagglutinating, precipitating, and blocking antibodies were found in this fraction, it was speculated that these actions were attributable to the same molecular species.[9] On this basis, it seemed probable that precipitating antibodies were contained in the antisera of all treated patients in the present study. However, preliminary studies on several whole Pool C antisera concentrated (by pervaporation) to yield hemagglutination titers of the order of 20,480 failed to reveal the presence of precipitins by means of microimmunoelectrophoresis.[13]

Studies have been started to fractionate all Pool C antisera with an aim to establish relations between the precipitins, hemagglutinating antibodies, and blocking antibodies.

SUMMARY

1. Precipitating antibodies have been revealed by microimmunoelectrophoretic analyses of unconcentrated and undiluted sera of 2 ragweed-sensitive subjects who had been treated with a purified dwarf ragweed fraction, Pool C. In addition to forming precipitin bands with the homologous antigen fraction, both antisera produced precipitin reactions with various other ragweed extracts. Precipitin patterns produced by the antisera of the 2 subjects with regard to a particular antigen extract were similar.

2. Pre-treatment and post-treatment sera of both subjects possessed the highest tanned cell (sensitized with Pool C) hemagglutination titers of all subjects (13 ragweed-sensitive, 12 not ragweed-sensitive) employed in the study. Multi-injection therapy with Pool C not only increased the Pool C titer but tended to increase the titers to whole ragweed extracts. Heating the Pool C antisera for 4 hours at 56° C. did not appreciably alter the hemagglutination titers but did diminish or abolish the precipitin reaction.

3. Although both subjects whose whole sera produced precipitin reactions obtained clinical relief during the ragweed season, relief was not complete.

ADDENDUM

One hundred and sixty days after post-treatment, the hemagglutination titers of Patients J and S declined to 640. Precipitin bands were not detected by microimmunoelectrophoresis.

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REFERENCES

1. Feinberg, S. M.: Allergy in Practice, ed. 2, Chicago, 1946, The Year Book Publishers, Inc., p. 73.
2. Criep, L. H.: Clinical Immunology and Allergy, New York, 1962, Grune & Stratton, Inc., pp. 132-142.
3. Sherman, W. B.: Changes in Serological Reactions and Tissue Sensitivity in Hay Fever Patients During the Early Months of Treatment, J. Immunol. **40**: 289, 1941.
4. Gordon, J., Rose, B., and Sehon, A. H.: Detection of Non-Precipitating Antibodies in Sera of Individuals Allergic to Ragweed Pollen by an In Vitro Method, J. Exper. Med. **108**: 37, 1958.
5. Feinberg, R. J., Davison, J. D., and Flick, J. A.: The Detection of Antibodies in Hay Fever Sera by Means of Hemagglutination, J. Immunol. **77**: 279, 1956.
6. Follensby, E. H.: The Applicability of In Vitro Tests in the Measurement of Neutralizing Antibody for Ragweed Pollen Extract, J. ALLERGY **29**: 103, 1958.
7. Feinberg, R., and Cline, J.: Studies on a Precipitin Reaction With Human Antibody to Ragweed, Fed. Proc. **19**: 212, 1960.
8. Perelmutter, L., Lea, D. J., Freedman, S. O., and Sehon, A. H.: Demonstration of Precipitating Antibodies in Sera of Ragweed Allergic Individuals by the Agar Gel Technique, Internat. Arch. Allergy **20**: 355, 1962.
9. Perelmutter, L., Lea, D. J., Freedman, S. O., and Sehon, A. H.: Determination of Rabbit and Human Anti-Ragweed Antibodies by the Precipitin Method, Internat. Arch. Allergy **21**: 1, 1962.
10. Friedman, H., and Criep, L. H.: Effect of Repository Therapy on Immunologic Responses to Pollen Extract, J. ALLERGY **33**: 215, 1962.
11. Goldfarb, A. R., and Callaghan, O. H.: Fractionation of the Antigens From Dwarf Ragweed Pollen, J. Immunol. **86**: 83, 1961.
12. Callaghan, O. H., and Goldfarb, A. R.: Isolation and Properties of an Allergen From Dwarf Ragweed Pollen, J. Immunol. **89**: 612, 1962.
13. Spitzer, R. H., and Kaplan, M. A.: (Unpublished data).
14. Scherr, G. H.: A New Alginate Adjuvant—Physical and Chemical Properties, Bact. Proc., p. 158, 1962.
15. Scherr, G. H., and Markowitz, A. S.: A New Alginate Adjuvant, Preliminary Release by Consolidated Laboratories, Chicago Heights, Ill.
16. Kaplan, M. A., Spitzer, R. H., Gutman, A. A., and Kalayil, P. K.: Hyposensitization Studies With a Purified Dwarf Ragweed Fraction, Ann. Allergy (In press).
17. Boyden, S. V.: The Absorption of Protein on Erythrocytes Treated With Tannic Acid and Subsequent Hemagglutination by Anti-Protein Sera, J. Exper. Med. **93**: 107, 1951.
18. Arbesman, C. E., Rose, N. R., Kantor, S. Z., and Beede, R. B.: Immunologic Studies of Ragweed-Sensitive Patients. I. Specificity and Sensitivity of Hemagglutination Reactions, J. ALLERGY **31**: 317, 1960.
19. Hirschfeld, J.: Immunoelectrophoresis—Procedure and Application to the Study of Group-Specific Variations in Sera, Science Tools **7**: 18, 1960.
20. Laurell, C. B., Laurell, S., and Skoog, N.: Buffer Composition in Paper Electrophoresis, Clin. Chem. **2**: 99, 1956.
21. Connell, J. T., Sherman, W. B., and Myers, P. A.: Antibody Studies in Constitutional Reactions Resulting From Injections of Ragweed Pollen Extract, J. ALLERGY **33**: 365, 1962.