

BIOLOGIC PROPERTIES OF A NON-PRECIIPITATING ANTIGEN FROM TIMOTHY POLLEN EXTRACTS¹

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The isolation and some of the chemical and immunochemical properties of a low molecular weight fraction of timothy pollen extracts were previously described (1-3). The results of these studies indicated that the isolated fraction inhibits the specific precipitation of rabbit anti-timothy sera and timothy antigens, fails to induce in either rabbits or guinea pigs antibody that is precipitable with the low molecular weight fraction, and shows slight direct skin test activity in timothy-sensitive patients. The following is an extension of this work which will show the antigenic relationship of the low molecular weight fraction (antigen D) and the major allergens of timothy pollen, provide further evidence of the haptenic properties of antigen D, and demonstrate that upon prolonged immunization with antigen D in complete Freund's adjuvant some rabbits produce antibody precipitable by one of the purified allergens (allergen B) of timothy pollen. None of the animals immunized produced antibody precipitable with antigen D.

MATERIALS AND METHODS

Preparation of antigens. Figure 1 represents a summary of the isolation methods used to obtain the various antigens utilized in these experiments. Detailed description of the methods of isolating antigen D and allergens A and B is given elsewhere (2, 4, 5).

Preparation of antisera. Rabbit anti-timothy sera were obtained by immunization with the nondialyzable residue of the aqueous extract of timothy pollen (WST) in complete Freund's adjuvant. Each animal received four simultaneous subcutaneous injections of 0.25 ml of the mixture (containing 10 mg WST protein/ml) in the neck and back areas at weekly intervals for 3 weeks. The animals were bled 3 weeks after

the last injection and thereafter at weekly intervals for 4 weeks. The resulting antisera were pooled and contained 1.8 mg of precipitating antibody/ml as determined by quantitative precipitin analysis (6) with the nondialyzable antigen material (WST).

Guinea pig anti-timothy sera were obtained by immunization with 10 mg of protein/ml of WST in complete Freund's adjuvant. Each animal received 1 ml of the mixture intraperitoneally at weekly intervals for 3 weeks. The animals were bled 3 weeks after the last injection. The resulting antisera were pooled and contained 2.5 mg of precipitating antibody/ml.

Passive cutaneous anaphylaxis (PCA) as described by Ovary (7), using 250- to 320-g Hartley strain guinea pigs, was performed with either rabbit or guinea pig anti-timothy sera. Animals were sensitized in the usual manner, and after a suitable latent period (4 hr), the minimal eliciting dose of antigen was determined. Inhibition of PCA by antigen D was performed as follows. Four hours after sensitization each animal was challenged by an intravenous (i.v.) injection of 500 μ g protein of antigen D. Animals challenged by antigen D gave no PCA reaction and were rechallenged 20 min later with 50 μ g protein of allergen B, and the degree of reaction was noted 30 min after the last injection. Control animals received an i.v. injection of 0.85% saline and 20 min later 50 μ g protein of allergen B.

Positive PCA reactions were graded as follows: 4+, 20 mm diameter or greater; 3+, 15 to 20 mm diameter; 2+, 10 to 15 mm diameter; 1+, 5 to 10 mm diameter.

Twenty-five Hartley strain guinea pigs (250 to 350 g) were immunized with either 10 mg protein/ml of antigen D or the crude pollen extract (WST) in complete Freund's adjuvant. Four weeks later the 5 animals immunized with antigen D and 10 of the animals sensitized

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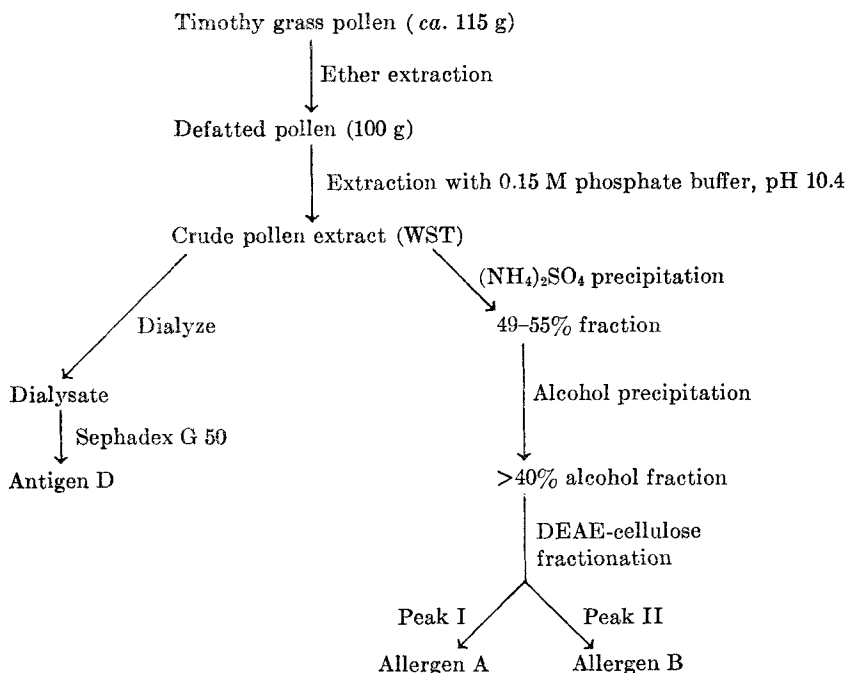


Figure 1. Isolation methods of antigen D and the allergens of timothy pollen (WST).

with WST were challenged with their respective immunizing antigens to induce anaphylaxis. To evaluate the ability of antigen D to inhibit anaphylaxis, the remaining WST sensitized animals were given an intravenous injection of antigen D, and 20 min later each animal received a second i.v. injection of 1.75 mg protein of WST.

Serial dilution of antigen D, WST and the purified allergens of timothy pollen in concentrations ranging from 10^{-3} to 10^{-11} g of protein/ml were prepared, and direct skin tests were conducted on timothy-sensitive patients by means of 0.01-ml intradermal injections.

An attempt was made to induce antibody directed against antigen D by immunizing rabbits with an antigen D-human serum albumin (D-HSA) conjugate prepared by a carbodiimide (1-cyclohexyl-3-(2-morpholinyl-4)-ethyl) carbodiimide metho *p*-toluene sulfonate) procedure (8). The resulting antigen D-HSA conjugate contained 1.75 moles of antigen D/mole of HSA. Five rabbits were injected at weekly intervals for 3 weeks with 10 mg protein/ml of the conjugate in complete Freund's adjuvant and bled 4 weeks after the last injection and at weekly intervals for the next 4 weeks.

Antigen D was adsorbed to a preparation of

methylated bovine serum albumin (MeBSA) (9), and the antigen D-MeBSA complex was used to immunize six rabbits as described by Plescia (10). The rabbits were immunized with the antigen D-MeBSA complex in complete Freund's adjuvant (resulting suspension contained 10 mg protein/ml of antigen D) at weekly intervals for 3 weeks, and the animals were bled 4 weeks after the last injection and at weekly intervals for the next 4 weeks.

Double diffusion analysis (11) with guinea pig anti-timothy sera was used to demonstrate the antigenic relationship of allergens A and B and the relationship of antigen D to these allergens. Nitrogen analyses were carried out by the Nessler method as described by Campbell *et al.* (12).

RESULTS

The antigenic relationship of allergens A and B was demonstrated by immunodiffusion of 1 mg protein/ml of allergen against guinea pig anti-timothy sera (Fig. 2A). A line of partial identity between the two allergens indicated that allergen A contains at least one antigenic determinant not shared with allergen B.

The relationship of antigen D, isolated from

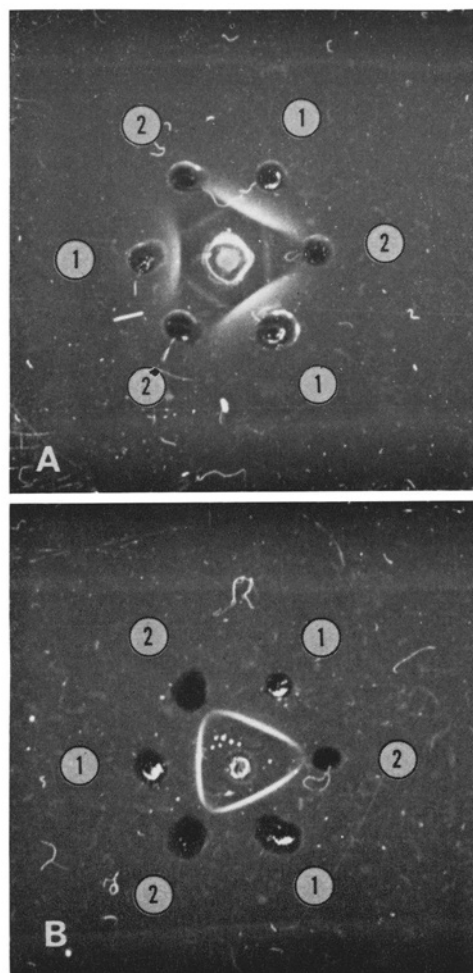


Figure 2. Antigenic relationship of antigen D and the allergens of timothy pollen. A, the center well contains guinea pig anti-timothy sera. The outer wells one and two contain 1 mg protein/ml of allergen A and B respectively. B, the center well contains guinea pig anti-timothy sera previously incubated with 10 mg antigen D at room temperature for 1 hr. The outer wells one and two contain allergens A and B in the same concentration and location as above.

timothy pollen, to allergens A and B was shown by the following experiment (Fig. 2B): Antigen D (10 mg protein in 0.1 ml) was incubated with guinea pig anti-timothy serum at room temperature for 1 hr before its addition to the center well of an immunodiffusion plate. The outer wells were filled with allergens A and B (1 mg protein/ml). Previous incubation of the guinea

pig anti-timothy sera with antigen D completely removed the line of precipitation of allergen B. Incubation of guinea pig antisera with either 0.1 ml saline or 10 mg bovine serum albumin did not remove the line of precipitation of allergen B. The data suggest that antigen D is antigenically closely related to allergen B and only slightly related to allergen A.

Attempts to elicit PCA reactions by means of either rabbit or guinea pig antisera directed against the antigens present in the crude timothy pollen extracts (WST) with as much as 40 mg protein of antigen D were unsuccessful. On the other hand, 0.5 mg protein of WST elicited strong PCA reactions. Only slight inhibition of PCA by antigen D was achieved when rabbit anti-timothy sera and WST were used as the sensitizing and challenging materials. Subsequent studies showed that the major antigenic material in WST for rabbits and guinea pigs is allergen A, and, as mentioned earlier, antigen D is antigenically only slightly related to this antigen. When the challenging antigen was allergen B, the degree of inhibition of PCA by antigen D was much more pronounced. Twelve animals received intradermal injections of guinea pig anti-timothy sera. Six of the animals received an i.v. injection of 500 μ g protein of antigen D 20 min before an i.v. injection of 50 μ g protein of allergen B. The remaining six animals received only a single injection of 50 μ g protein of allergen B. The results (Table I) show that the six animals receiving only an injection of allergen B gave positive PCA reactions at a serum concentration of 0.05 μ g antibody nitrogen. The six animals challenged initially with antigen D and rechallenged with allergen B gave significantly weaker reactions. None of the six animals gave positive PCA reactions when challenged with antigen D, and three of the six animals were completely protected by the previous injection of antigen D. Attempts to inhibit PCA reactions by earlier injection of antigen D when the challenging antigen was allergen A have shown only slight inhibition.

Evidence of inhibition of systemic anaphylaxis by antigen D is presented in Table II. Guinea pigs immunized with antigen D in complete Freund's adjuvant did not show any signs of anaphylaxis upon i.v. challenge with either 10 mg protein of antigen D or 1.75 mg protein of WST. On the other hand, eight of ten animals

TABLE I

Inhibition by antigen D of passive cutaneous anaphylaxis with guinea pig antisera and allergen B

Antigen	Protein Concentration	No. of Animals	Ab N/injection (μ g)						
			3.2	1.6	0.8	0.4	0.2	0.1	0.05
Allergen B	μ g 50	6	3+ ^a	3+	2+	2+	+	+	+
Antigen D (1st injection)	500	6	—	—	—	—	—	—	—
Allergen B (2nd injection 20 min after 1st injection)	50	3	—	—	—	—	—	—	—
		2	2+	+	+	$\pm$	$\pm$		
		1	2+	+	+	+	+	+	+

^a Diameter of the reactions were graded $\pm$ less than 5 mm; +, 5 to 10 mm; 2+, 10 to 15 mm; 3+, 15 to 20 mm; 4+, greater than 20 mm; —, negative.

TABLE II

Anaphylaxis in guinea pigs with timothy pollen (WST) fractions

No. of Animals	Sensitizing Antigen	Challenge Antigen	Protein Concentration		Response
			mg		
5 ^a	Antigen D	Antigen D	10	5/5	No reaction
		WST	1.75	5/5	No reaction
10	WST	WST	1.75	8/10	Died
				2/10	Moderate reaction
10 ^a	WST	Antigen D	10	10/10	No reaction
		WST	1.75	8/10	No reaction
				1/10	Severe reaction 5 min later
				1/10	Moderate reaction 6 min later

^a Animals rechallenged 20 min later with WST.

immunized with WST showed marked anaphylaxis and died within 2 min following an i.v. challenge with WST. The remaining two animals showed moderate immediate reactions and died within a few hours. To ascertain if antigen D inhibits anaphylaxis, a group of 10 guinea pigs immunized with WST received an i.v. injection of 10 mg protein of antigen D. None of these animals showed any signs of anaphylaxis, and 20 min later they received an i.v. challenge of 1.75 mg protein of WST. Eight of ten animals showed no anaphylaxis, and the remaining two animals showed varying degrees of anaphylaxis and died within several hours. The administration of either saline or 10 mg of bovine serum

albumin 20 min before the injection of WST did not protect sensitized animals from anaphylaxis.

Further confirmation of the antigenic relationship of antigen D and allergens A and B was demonstrated by examination of the sera of animals extensively immunized with antigen D in complete Freund's adjuvant. The sera of these animals showed no demonstrable antibody directed against antigen D (PCA and ring tests) but showed varying degrees of reactivity with allergen B and WST. The sera of animals immunized with antigen D either alone or coupled to human serum albumin in complete Freund's adjuvant after prolonged stimulation (6 to 8

weekly injections) showed positive ring tests with 0.1 mg protein/ml of either allergen B or WST 3 weeks after the last injection but negative ring tests with the same concentration of allergen A. Attempts to restimulate animals producing anti-allergen B antibody by further injections of antigen D or the antigen D-human serum albumin conjugate were not successful. The sera of animals immunized with antigen D adsorbed to methylated bovine serum albumin (antigen D-MeBSA) also showed no demonstrable antibody directed against antigen D but showed positive responses (PCA and ring test) with allergens B and WST. Animals immunized with antigen D-MeBSA responded to restimulation, and several animals produced 1 to 2 mg protein of anti-allergen B antibody/ml.

Antigen D has been used in direct skin testing of timothy-sensitive patients by several clinical allergists. The results (2, unpublished observations) of this testing in over 100 patients clearly indicate that antigen D possesses some degree of skin reactivity in those timothy-sensitive patients tested. In general antigen D has a lower degree of skin reactivity than the crude pollen extract and considerably less than the purified allergens A or B.

DISCUSSION

Rabbits and guinea pigs extensively immunized with antigen D alone, coupled to human serum albumin or adsorbed to methylated bovine serum albumin in complete Freund's adjuvant, failed to produce antibody demonstrable with antigen D directly by precipitation, PCA or anaphylaxis. However, the sera of these animals did contain varying amounts of antibody that were precipitable with one of the purified allergens of timothy pollen (allergen B). The close antigenic relationship of antigen D and allergen B was further established by the inhibition both of precipitation in agar gel and PCA of either rabbit or guinea pig anti-timothy sera with allergen B (Fig. 2B and Table I respectively).

Although antigen D cannot strictly be considered a hapten, it does possess haptenic properties as demonstrated by its inhibition of PCA, anaphylaxis and precipitin reactions of both guinea pig and rabbit anti-timothy sera and timothy pollen antigens. Efforts to elicit with antigen D any direct immunologic reaction in timothy (WST) sensitized animals have been

completely unsuccessful. Only in the direct skin testing of timothy-sensitive patients and the induction of antibody demonstrable with the antigenically related allergen B does one find evidence of the antigenic character of antigen D.

The major antigenic material of WST is allergen A; antigen D is not antigenically closely related to this allergen. One would have expected that despite a previous injection of antigen D, a larger number of animals would have undergone a fatal anaphylactic reaction when challenged with WST. The observation that antigen D inhibited anaphylaxis to a greater extent than PCA when the challenging antigen was WST may be due to a quantitative difference in the amount or type of antibody required to evoke these reactions.

Several investigators (13-16) have demonstrated that bivalent antigens are capable of eliciting PCA reactions. The inability of antigen D to elicit PCA reactions, anaphylaxis or precipitate with either rabbit or guinea pig anti-timothy sera suggests that antigen D acts as a monovalent fragment of allergen B. The molecular weight of allergen B determined by the sedimentation-diffusion method in the model E analytical ultracentrifuge is 10,500. Estimation of the molecular weight of antigen D has been made by comparison of the elution volumes of antigen D and the trinitrophenyl (TNP) derivatives of insulin (B chain), adrenocorticotrophic hormone (ACTH) and bacitracin of low coupling ratios (2 to 4 TNP groups/mole on Sephadex G 50 fine in 0.1 M NaHCO₃, pH 8. Allergen B is not retained on Sephadex G 50 and is eluted with the void volume. However, antigen D and TNP-ACTH were retained on Sephadex G 50 and eluted from the column (1 by 40 cm) with the same elution volume. The results suggest that the molecular weight of antigen D is near 5000, and sedimentation-equilibrium studies are in progress to confirm this molecular weight. Comparison of the amino acid composition of antigen D (3) with preliminary amino acid analysis of allergen B by means of the methods of Spackman, Stein and Moore (17) suggests that allergen B is not a simple dimer of antigen D despite the apparent relationship of their molecular weights.

The relatively low skin reactivity of the non-precipitating antigen in timothy-sensitive pa-

tients, its ability to inhibit anaphylaxis in guinea pigs and its antigenic relationship to the major allergens of timothy pollen suggest that the nonprecipitating antigen may provide an alternative approach in the treatment of timothy-sensitive patients. Desensitization of timothy-sensitive patients with the nonprecipitating antigen could progress more rapidly since a patient could safely be given larger doses at each visit than is currently the practice with crude pollen extract. With this in mind, a pilot study in collaboration with Dr. Frank Perlman of the University of Oregon Medical School is being conducted to determine the merits of the nonprecipitating antigen in the desensitization of timothy-sensitive patients.

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SUMMARY

The close antigenic relationship of antigen D and allergen B was demonstrated by inhibition of both passive cutaneous anaphylaxis (PCA) and precipitation in agar gel. The antigenic relationship of allergen A and B was demonstrated by a line of partial identity in micro-double diffusion analysis.

Antigen D possesses haptenic properties as demonstrated by its inhibition of PCA, anaphylaxis and precipitin reactions of either rabbit or guinea pig antisera to timothy pollen antigens.

The antigenic character of antigen D is demonstrated by its direct positive skin reactions in timothy-sensitive patients and by its induction

in rabbits with varying amounts of antibody demonstrable with allergen B but not with antigen D.

REFERENCES

1. Malley, A. and Campbell, D. H., *Fed. Proc.*, **22**: 560, 1963.
2. Malley, A., Campbell, D. H. and Heimlich, E. M., *J. Immun.*, **93**: 420, 1964.
3. Malley, A., Saha, A. and Campbell, D. H., *Immunochemistry*, **1**: 237, 1964.
4. Malley, A., Reed, C. E. and Lietze, A., *J. Allerg.*, **33**: 84, 1962.
5. Malley, A. and Dobson, R. L., *Fed. Proc.*, **25**: 729, 1966.
6. Campbell, D. H., Garvey, J. S., Cremer, N. E. and Sussdorf, D., *Methods in Immunology: A Laboratory Text for Instruction and Research*, p. 136, W. A. Benjamin, Inc., New York, 1963.
7. Ovary, Z., *Immunological Methods*, Edited by J. F. Ackroyd, p. 259, Blackwell Scientific Publications, Oxford, England, 1964.
8. Sheenan, J. C. and Hlauka, J. J., *J. Org. Chem.*, **21**: 439, 1956.
9. Sweeka, N. and Cheng, T. Y., *J. Molec. Biol.*, **4**: 151, 1962.
10. Plescia, O., *Proc. Nat. Acad. Sci. U.S.A.*, **52**: 279, 1964.
11. Ouchterlony, O., *Progress in Allergy*, Vol. 5, p. 1, S. Karger AG, Basel, Switzerland, 1958.
12. Campbell, D. H., Garvey, J. S., Cremer, N. E. and Sussdorf, D., *Methods in Immunology: A Laboratory Text for Instruction and Research*, p. 53, W. A. Benjamin, Inc., New York, 1963.
13. Ovary, Z., *C. R. Acad. Sci.*, **253**: 582, 1961.
14. Abuelo, J. G. and Ovary, Z., *J. Immun.*, **95**: 113, 1965.
15. Landsteiner, K. and van der Scheer, J., *J. Exp. Med.*, **57**: 633, 1933.
16. Levine, B. B., *J. Immun.*, **94**: 111, 1965.
17. Spackman, D. H., Stein, W. H. and Moore, S., *Anal. Chem.*, **30**: 1190, 1958.