

THE CHEMICAL CHARACTERIZATION OF A MAJOR ALLERGEN OF TIMOTHY POLLEN*

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(Received 12 July 1974)

Abstract—Bio-Gel P-2 filtration of timothy dialysate fractions resulted in the isolation of a yellow pigmented fragment, antigen D₃, with a mol wt near 1000. The relationship of antigen D₃ to the antigenic and allergenic determinants of allergen B was demonstrated by its significant inhibition (77 per cent) of PCA reactions in guinea pigs and 78 per cent inhibition of WST-induced histamine release reactions from sensitized monkey lung tissue. Preliminary characterization of the antigen D₃ fragment suggests that its major components are a flavonoid pigment (quercetin), a disaccharide moiety (cellobiose) linked to threonine (serine) by O-glycosidic bonds

INTRODUCTION

Although several allergens have been isolated to very high stages of homogeneity attempts to identify the chemical structure of these allergenic determinants have been limited to the studies of Elsayed *et al.* (1971, 1971a), Berrens (1970) and more recently Mole *et al.* (1974). Previous studies (Malley *et al.*, 1967) demonstrated that the dialysate fraction of timothy pollen extracts contained a low mol wt fragment (antigen D) that is structurally closely related to the major allergen of timothy pollen (allergen B). Gel-filtration of the timothy dialysate fraction on Sephadex G-25 revealed two smaller fragments antigen D₂ (approx 2500 mol. wt) and antigen D₃ (approx 1000 mol. wt) that specifically inhibit antigen-induced passive cutaneous anaphylaxis reactions in guinea pigs. This manuscript describes the isolation of antigen D₃ by Bio-Gel P-2 gel-filtration, the relationship of antigen D₃ to the major allergen of timothy pollen (allergen B), and preliminary chemical characterization of the antigen D₃ fragment.

MATERIALS AND METHODS

Antigens A detailed description of the methods used in preparing the aqueous extract of timothy pollen (WST), allergens A and B, and antigen D from the dialysate fraction of timothy pollen extracts is described elsewhere (Malley *et al.*, 1967). A crude extract of ragweed pollen (5 mg protein/ml) was obtained from Hollister-Stier Labs, Spokane, Wash, and ragweed antigen E (10 mg protein/ml) was provided as a gift by Dr J. George Center, Center Labs., Port Washington, N.Y.

Sera Rabbit antitimothy sera was prepared as previously described (Malley *et al.*, 1967) and contained 1.8 mg antibody protein per ml. Rabbit anti-BSA was prepared as described by Campbell *et al.* (1964) and contained 2.1 mg antibody protein per ml.

Serum from a timothy-sensitive patient (A.G.-PK titer of 5000) was obtained from Dr F. Perlman (Portland, Oregon). Serum from a ragweed-sensitive patient (J.B.-PK titer of 1000) was obtained from Dr. C. D. May, National Jewish Hospital, Denver, Colorado.

Gel-filtration The aqueous extract of timothy pollen was dialyzed against distilled water using Spectrophore 1 dialysis membrane (exclusion limit 6000-8000), Spectrum Medical Industries, Los Angeles, Calif., at 4°C for 24 hr. The dialysate fraction was lyophilized to dryness, redissolved in a minimum of distilled water and stored in small aliquots at -20°C until used. The dialysate fraction (80-120 mg protein/5 ml) was applied to a Bio-Gel P-2 column (2.5 × 100 cm) equilibrated with distilled water. The proteins eluted with distilled water (36 ml/hr) were monitored by optical density (LKB adsorptionmeter) and the isolated fractions were concentrated by lyophilization.

Passive cutaneous anaphylaxis

Passive cutaneous anaphylaxis (PCA) as described by Ovary (1964) was performed using rabbit antitimothy serum. The inhibition of PCA was performed as follows: 3 hr after sensitization animals were challenged with 10 units of antigen D₃ (1 unit arbitrarily chosen as OD₄₀₄ of 0.1). Twenty minutes later each animal was challenged by intravenous (i.v.) injection of 160 µg protein of allergen B. 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 160 µg protein of allergen B. Reactions were graded as follows: 4+, greater than 20 mm diameter; 3+, 16-20 mm diameter; 2+, 11-15 mm diameter; +, 5-10 mm diameter; ±, less than 5 mm diameter; -, negative.

Allergen induced histamine release

Histamine release from monkey lung tissue passively sensitized with the serum of allergic patients was performed as previously described (Malley *et al.*, 1968).

Chemical analysis of antigen D₃

N-terminal analysis of antigen D₃ was performed according to the method of Fraenkel-Conrat *et al.* (1955). Ether soluble DNP-amino acids obtained following acid hydrolysis (5.7 N HCl for 20 hr at 105°C) were evaluated by thin-layer chromatography (TLC) on silica gel plates (Brinkman Inst. Co., Westbury N.Y.) in toluene:pyridine:2-chloroethanol:0.8 N NH₃ (5:1.5:3:3) and CHCl₃:benzyl alcohol:acetic acid (70:30:3).

Carbohydrate analysis of antigen D₃ was evaluated by both acid and alkaline hydrolysis. Partial acid hydrolysis of antigen D₃ was carried out in 1 M H₂SO₄ at 100°C for 7 hr as previously described (Malley *et al.*, 1964). Alkaline hydrolysis was carried out in saturated aqueous

* This work was supported by USPH Grant AI 06274, AI 09234, and PR 00163

Ba(OH)₂ for 10 hr at 60°C. Excess Ba⁺⁺ was removed by the addition of 1 M H₂SO₄. The resulting precipitate was removed by centrifugation at 3000 rev/min for 20 min at 4°C, and the supernatant used to determine the carbohydrate moiety of antigen D₃. The carbohydrate fractions obtained by Dowex 50 × 2 chromatography (Malley *et al.*, 1964) of acid or alkaline hydrolysates were studied by TLC on silica glass plates in butanol acetic acid water (4:1:5) and butanol pyridine water (3:1:1) and compared with R_F values of known sugar standards (Sugar Standard kit, Consolidated Labs, Inc., Chicago Heights, Ill.) run simultaneously.

The yellow pigment associated with the antigen D₃ obtained following either acid or alkaline hydrolysis was eluted from a Dowex 50 × 2 column (0.5 × 4 cm) with 5 M NH₄OH. The pigment in the effluent was adsorbed on Norite A, washed several times with water, and eluted from the Norite A with 1 M NaOH. The filtered pigment was studied in a Cary Model 15 recording spectrophotometer.

RESULTS

Bio-Gel P-2 filtration (Fig. 1) not only results in a good recovery of antigen D but also permits the detection of antigen D₃ (peak V) due to the yellow pigmentation of this fraction. Repeated chromatography of the antigen D₃ fraction on the P-2 column resulted in elution at the same volume without evidence of higher mol. wt contaminants. Chromatography of DNP₂-Bacitracin prepared according to the method of Mancino *et al.* (1970) eluted with a volume between 950 and 1100 ml suggesting that antigen D₃ has a mol wt near 1000.

The relationship of antigen D₃ to the antigenic determinant expressed on allergen B was evaluated

by PCA experiments in guinea pigs (Table 1). Control animals injected i.v. with 160 µg protein of allergen B gave positive PCA reactions (7 × 9 mm diameter) at a serum concentration of 0.04 µg antibody nitrogen. Animals challenged with 10 units of antigen D₃ prior to being injected with allergen B did not show positive PCA reactions at any concentration of antibody tested. These animals upon allergen B challenge gave significantly weaker reactions compared to control animals. Two of the six animals were completely protected by the previous injection of antigen D₃, and the remaining four animals gave weak positive reactions (5 × 6 mm) at the 0.3 µg antibody nitrogen level. Increasing the duration of time between the injection of antigen D₃ and allergen B challenge to one hour greatly reduced the ability of antigen D₃ to inhibit allergen B-induced PCA reactions, and after a 3 hr latent period antigen D₃ was not inhibitory. Specificity of the inhibitory properties of antigen D₃ was seen by the failure of antigen D₃ to inhibit PCA reactions of animals sensitized with rabbit anti-BSA serum and challenged with BSA.

The relationship of antigen D₃ to the major allergenic determinant of timothy grass pollen was evaluated using the method of allergen-induced histamine release from monkey lung tissue passively sensitized with the serum of a timothy-sensitive patient (Table 2). Sensitized tissue treated with Tyrode's solution prior to allergen challenge released 2.64 µg of histamine/g tissue upon incubation with WST. On the other hand, preincubation of sensitized tissue with varying concentrations of antigen D₃ gave a significant inhibition (78 per cent) of allergen-reagin induced histamine release upon challenge with WST. Sensitized tissue challenged directly with antigen D₃ (10 units) did not result in the release of histamine. Specificity of the antigen D₃ inhibition was seen by the failure of antigen D₃ to inhibit allergen-reagin induced histamine release reactions of tissue sensitized with the serum of a ragweed-sensitive patient and challenged with either ragweed extract or ragweed antigen E.

The carbohydrate moiety obtained by Dowex 50 × 2 chromatography following partial acid and alkaline hydrolysis of antigen D₃ was evaluated by ascending chromatography using silica gel thin layer plates and showed that the sugar moiety obtained following a 7 hr alkaline hydrolysis had R_F values (0.181 and 0.237 respectively) in butanol:acetic acid water and butanol:pyridine:water corresponding to the glucose standard. In addition, chromatography of a mixture of approximately equal quantities of the 7 hr alkaline

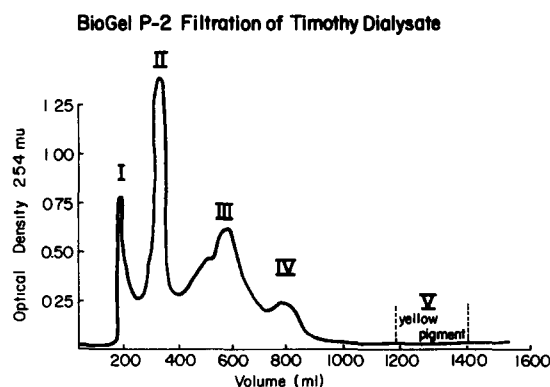


Fig. 1 Bio-Gel P-2 filtration of timothy dialysate. Fraction I and V represent antigen D and antigen D₃ respectively.

Table 1 Inhibition by antigen D₃ of PCA with rabbit anti-WST antisera and allergen B

Antigen	Protein concentration	No of animals	Antibody N/site (µg)					
			1.3	0.6	0.3	0.15	0.08	0.04
Allergen B	160 µg	6	3+	3+	2+	2+	+	+
Antigen D ₃ (1st inj)	10 units ^a	6	—	—	—	—	—	—
Allergen B (2nd inj 20 min later)	160 µg	2	±	—	—	—	—	—
		3	2+	+	+	—	—	—
		1	2+	2+	+	—	—	—

^a 1 unit represent OD₄₀₄ = 0.1

Table 2. Antigen D₃ inhibition of timothy allergen–reagin induced histamine release from passively sensitized monkey lung tissue

Tissue sensitization ^a	Treatment	Concentration	Allergen ^b challenge	Histamine release (μg/g)	% Inhibition
A G.	Tyroides	—	WST	2.64	—
A.G	Antigen D ₃	1 unit ^d	WST	1.12	58
A G	Antigen D ₃	5 units	WST	0.80	70
A G	Antigen D ₃	10 units	WST	0.61	78
A G	Antigen D ₃	10 units	—	0	—

^a Tissue incubated for 2 hrs at 37°C with the serum of a timothy-sensitive patient (P-K titer-5000)

^b Suspension incubated with 40 μg protein WST for 15 min at 37°C

^c Sensitized tissue incubated for 10 min at 37°C with antigen D₃ prior to allergen challenge

^d 1 unit of antigen D₃ represent OD₄₀₄ = 0.1.

hydrolysate and the glucose standard in both solvents yielded a single spot. In contrast the sugar moiety obtained by acid hydrolysis of the antigen D₃ fragment had *R_F* values (0.055 and 0.156 respectively) in the same solvents corresponding to a cellobiose standard.

The yellow pigment isolated from either the acid or alkaline hydrolysates of antigen D₃ and liberated from Norite A gave adsorption maximum peaks at 275 and 404 nm. The pigment exposed to ultraviolet light gives a yellow fluorescence which increases in intensity upon exposure to an NH₃ vapor suggesting that the yellow pigment possesses a flavonol structure similar to quercetin.

Two dimensional TLC of the acid hydrolysate of FDNB treated antigen D₃ revealed three faint spots and one strong yellow spot. Two of the faint yellow spots corresponded in *R_F* values to 2,4-dinitrophenol and 2,4-dinitroaniline. The third faint yellow spot corresponded in *R_F* value to DNP-serine, and the intense yellow spot corresponded to DNP-threonine. In addition, TLC of the acid hydrolysate (57 *N* HCl for 20 hr at 105°C) of antigen D₃ in butanol:acetic acid: water (4:1:5) and butanol pyridine:water (3:1:1) showed only two ninhydrin staining spots

DISCUSSION

The relationship of antigen D₃ to the allergenic determinant of timothy grass pollen was demonstrated by the significant inhibition (78 per cent) of WST-induced histamine release from sensitized lung tissue (Table 2). Although higher doses of antigen D₃ might have resulted in a greater inhibition of WST-induced histamine release a portion of the residual reactivity reflects the level of reagin directed against allergen A in the serum used to sensitize the lung tissue and the presence of allergen A in the challenging allergen (WST). Previous studies demonstrated (Malley *et al.*, 1967, 1970) a close antigenic relationship between antigen D and allergen B. The data presented in Table 1 would further suggest that antigen D₃ was also antigenically closely related to allergen B. Preinjection of 10 units of antigen D₃ 10–20 min prior to challenge with allergen B gave a 77 per cent inhibition of PCA. The reduction in antigen D₃ inhibition of PCA reactions upon increasing the duration between antigen D₃ injection and allergen B challenge probably reflects the avidity of antigen D₃–antibody interaction

Preliminary chemical characterization of antigen D₃ indicates the presence of a yellow flavonoid pigment, a sugar moiety, and at least one amino acid—threonine (serine). The sugar moiety of antigen D₃ isolated following alkaline hydrolysis was glucose and following acid hydrolysis was the diglucoside—cellobiose. The resistance of antigen D₃ to acid hydrolysis (1 *M* H₂SO₄) of the sugar moiety into glucose units suggests that cellobiose represents the native structure of the carbohydrate portion of antigen D₃. The yellow pigment associated with antigen D₃ has spectral and chromatographic characteristics of the flavonoid compound quercetin. Flavonoid pigments are widely distributed among plants and the flavonoid pigment dactylin has previously been reported to be a constituent of timothy pollen (Hampton *et al.*, 1955). Structural characterization of dactylin (Harborne, 1959) indicates that it contains a diglucoside moiety attached to the 3 position of the quercetin molecule through an ether linkage. Although the location of cellobiose attachment to the flavonol structure of antigen D₃ is not known the composition data of this fragment is clearly consistent with the structure of dactylin

Berrens has previously suggested that many atopic allergens possess spectral characteristics (adsorption maxima at 305–310 nm) similar to 1-amino-1-deoxy-2-ketose residues and incorporate sites of the ε-amino group of lysine-monosaccharide (N-glycoside) linkage (Berrens, 1970). Spectrophotometric characteristics of antigen D₃ demonstrate an adsorption maxima at 404 mμ rather than the 305–310 nm adsorption maxima reported to be associated with N-glycoside linkages. Furthermore, the resistance of antigen D₃ to partial acid hydrolysis, its susceptibility to mild alkaline hydrolysis, and the reactivity of the amino acid constituents of antigen D₃ with FDNB strongly suggest that the linkage of threonine and the sugar moiety is not an N-glycoside linkage. More consistent with the above observations is an O-glycoside linkage of threonine (serine) to the cellobiose moiety of antigen D₃. The recent review by Spiro on glycoproteins (1973) indicates that in most proteins containing O-glycosidic linkages both serine and threonine residues are usually found, and that a high content of proline in the immediate vicinity of the glycopeptide bond may play a role in shaping the region for serine and threonine glycosylation. Although proline was not found in association with antigen D₃ earlier studies regarding the amino acid composition of

antigen D (Malley *et al.*, 1964) indicated an unusually high concentration (23 per cent) of proline. The observation that both threonine and serine may be the N-terminal amino acid of antigen D₃ is also consistent with the observations made by Spiro and other investigators. The data presented in this manuscript suggests that the structure of antigen D₃ involves the linkage of threonine (serine), cellobiose, and the flavonoid pigment quercetin through ether linkages.

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