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Address:

T. P. King
The Rockefeller University
New York, NY 10021
U.S.A.

Timothy Pollen Allergens

HENNING LØWENSTEIN

University of Copenhagen, Copenhagen, Denmark

Among grasses timothy pollen allergens are the best studied. Through a series of studies Augustin et al. (1–4) characterized timothy pollen antigens and demonstrated the existence of allergens with molecular weight about 14,000 dalton and α to β mobilities. Malley et al. (12, 13) later partially purified one of these, denoted Antigen B.

An intensive study of timothy pollen allergens was initiated by Løwenstein, Weeke and co-workers (6, 7, 10, 11, 14, 16) after very powerful tools for the characterization of allergenic extracts — particularly QIE (8) and RAST-technique — but also the more sophisticated separation techniques were made available. During these studies timothy pollen extracts were revealed to be composed of at least 30 antigenic components of which 12 were shown by means of CRIE to react with patients' specific IgE (i.e. act as allergens). Four of these allergens were isolated, partially characterized by physical-chemical methods and their allergenic activities verified by direct *in vitro* and *in vivo* tests on patients.

During the past few years we have continued our studies on timothy pollen extract and have included it in a model for allergen standardization (9); it has also been studied in connection with a clinical comparison between two aluminium hydroxide adsorbed timothy pollen extracts: Alutard® SQ and the mixture of two

purified allergens designated antigens Nos. 19 and 25 (15).

It is the aim of this work to repeat the main results of our previous isolation studies and to relate this with our current studies — paying special attention to the identification of allergens by means of CRIE, and the importance of the isolated allergens in relation to clinical experiences.

Four allergens, antigen Nos. 3, 19, 25 and 30 (ag 19 and 25 always remained together) were isolated from freeze-dried aqueous extract of timothy pollen by various combinations of anionic and cationic exchange, and gel chromatography. The allergens were all of protein nature and contained less than 2% (w/w) immunochemically detectable impurities. Molecular weights and pI's were determined to $<10 \times 10^3$, 15×10^3 , 15×10^3 , 34×10^3 and 3.9, 4.9, 4.5, 9.4, respectively. Antigen Nos. 19 and 25 were determined to be the major components of antigen B.

The allergenic activities were verified by means of RAST inhibition and prick-tests and proved antigen No. 25 to be quantitatively the most important allergen of timothy pollen. Immunologically, this allergen was shown to be partially identical to the most important pollen allergen from three other grasses (7).

The main advantages of this purification study

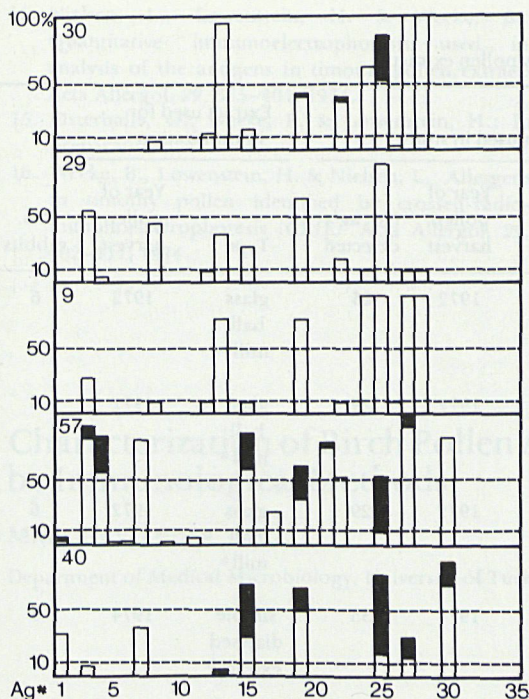


Fig. 1. Allergograms from five different CRIE-studies performed on timothy allergen extracts. The columns corresponding to the various classification numbers (below) indicate the percentage of patients whose IgE reacts to that allergen. Black parts of columns indicate strong IgE binding. Number in upper left corner indicates the number of patients studied.

compared to the previous ones were the simple methods of monitoring and detection of impurities, both based upon the use of rabbit antibodies as a stable chemical reagent in the QIE methods.

The isolation performed was based upon our knowledge of the allergenic composition of timothy pollen extracts from three previous CRIE-studies (8, 16) as well as analytical isolation studies which could predict the versatility of future isolations. The results of these CRIE-studies as well as two newly performed studies are given in Table 1 and Fig. 1. The classification numbers given to individual allergens are consistent with those originally published (14). The data were in all cases corrected for non-specific binding ^{125}I -aIgE by using the relevant control sera from non-timothy allergic patients. An arbitrary distinction has been made between "minor allergens", to which "less than 10% of the selected timothy allergic patients sera react": "major allergens", to which "more than 50% of the patients react and to which more than 25% of the patients show a

"strong binding of IgE"*; and those in between. Several antigens did not show any binding of specific IgE but can still be recognized as potential minor allergens although the probability of finding a patient reacting to them is very low. The allergens to which less than 10% of the patients reacted also varied considerably from study to study, as did the allergens in the intermediate groups. Only antigen No. 25 was found to be a major allergen in all studies. These results of course reflect the various compositions of the extract used for the studies and for the immunizations; the specificity and titre of the rabbit antibodies raised; and the patients studied. Nevertheless this study reveals a qualitative correlation between the various CRIE-studies performed. As stated earlier, CRIE is a qualitative method which can only be used semi-quantitatively for one allergen *per se*, provided exactly the same experimental conditions are present. The sensitivity of the method, i.e. the intensity of the radiostaining, depends on the number of sites of the precipitated antigen available for reaction with specific IgE and the affinity of the various specificities of IgE. The availability for IgE further depends on the density and the structure of the precipitate (i.e. number of sites on the antigen, molecular weight and affinity of rabbit antibodies).

Similarly, examples from the literature have shown that purified major allergens work as well as — or even better than, complex extracts in diagnoses; but we have no proof of a generally better effect of treatment with purified major allergens than with complex mixtures. This might be explained by the difference between triggering and immunization, since a general major allergen will of course always trigger the histamine-release from the patients' mast cells or basophils and bind patients' specific IgE in RAST. Thus, for optimal hyposensitization the individual mixture of the patient should preferably be used. For practical reasons it is not possible to-day to produce tailored extracts. Only a careful use of CRIE will provide the data necessary for selection of the optimal composed extract for treatment of the average allergic patient.

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* "Strong binding of IgE" is shown by precipitates which give rise to visible radiostaining on X-ray film after 3 days of exposure (8), or fall in CRIE class A, B or C according to Aukrust & Aas (5). No sharp limits exist since both methods are dependent on the patients selected for the study and the animal antibodies used in the immunoplates.

Table 1
CRIE-studies on timothy pollen extracts

Study		Patients			Extract used in study			Extract used for immunization		
No.	Ref.	Grass treated patients excluded	Year of blood sampling	n	Type	Year of pollen harvest	No. ag's detected	Type	Year of pollen harvest	No. rabbits
1	15	no	1973	30	glass ball mill ^a	1972	28	glass ball mill ^a	1972	6
2	8	yes	1974	29	simple dialysed extract ^b	1972	29	glass ball mill ^a	1972	6
3	8	yes	1974	9	simple dialysed extract ^b	1972	29	glass ball mill ^a	1972	6
4	9	yes	1976	59	simple dialysed extract ^b	1974	35	simple dialysed extract ^b	1974	3
5	16	yes	1978	40	soluprick® SQ ^c	1975	18	simple dialysed extract of 5 grasses ^b	1972	6

^aGlass ball homogenization of 1:10 (w/v) pollen suspension, 0°C, ionic strength 0.13, pH 8.2, 1 min.

^bExtraction of 1:10 (w/v) pollen suspension, 5°C, ionic strength 0.14, pH 7–8, 20 h: centrifugation, dialysis, freeze drying.

^cALK, Allergologisk Laboratorium, Copenhagen, Denmark.

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Address:

Henning Løwenstein
The Protein Laboratory
University of Copenhagen
Sigurdsgade 14 DK-2200
Copenhagen
Denmark

Characterization of Birch Pollen Allergens by Immunological Methods

MARKKU VIANDER

Department of Medical Microbiology, University of Turku, Turku, Finland

Birch pollen extract was prepared by extraction of 1.0 g non-defatted pollen (*Betula pendula seu verrucosa*) in 10 ml phosphate-buffered saline (pH 7.2) by mild shaking for 24 h and subsequent centrifugation at 22 000 g for 20 min at 4°C (10). The protein content and the allergenic activity of this extract were 7.9 mg/ml and 100%, respectively. Rabbit antiserum against birch pollen was raised and used in quantitative immunoelectrophoretic methods as described by Axelsen et al. (2). The crossed immunoelectrophoretic (CIE) precipitation pattern obtained at pH 8.6 in agarose gel revealed nine different antigens. No cathodically migrating antigens were observed. Crossed radioimmunoelectrophoresis (CRIE) indicated that IgE from patients allergic to birch had affinity for the major allergens (antigens Nos. 2 and 7/8). Fractionation of the allergen-containing extract by Sephadex G-100 gel filtration was followed by fused rocket immunoelectrophoresis (2) or enzyme-immunoassay (EIA; 9, 11) to obtain the immunochemical elution profile (Fig. 1). Antigens eluted in protein peaks A, B and C, with mean molecular weights of 79 000, 29 000 and 17 000 daltons, respectively, were the most effective in binding rabbit antibodies in EIA. Two thermostable (TS) antigens (Nos. 7/8, mw 10 000–20 000) and a thermolabile one (No. 2, mw 29 000) were shown by CIE and fused rocket immunoelectrophoresis. These results are in agreement with those of Belin (4). The concentration of the TS antigens in the pooled protein peaks was determined by rocket immunoelectrophoresis and the allergenic activity was shown both by prick-skin-testing and by the RAST inhibition method (12). The results are given in Table 1.

Pooled peak C fractions (mw 10 000–20 000; Fig. 1, Table 1) containing the main allergenic activity showed about 5-fold increase in the concentration of the TS antigen and about 2- to 5-fold increase in the specific allergenic activity (per µg of protein) when compared to the whole extract (9). About 30% of the total allergenic activity was recovered in the peak C fractions and less than 0.5% outside these fractions.

The specific allergenic activity of the peak B fractions was 10–300 times lower than that of the peak C fractions by prick skin testing and by the RAST inhibition method, respectively (9). The non-parallelity of the inhibition curves and the antigenic differences in CRIE indicate that peak B contains allergens distinct from those of peak C. In addition, peak B certainly contains some of the allergens of peak C as cross-contaminating antigens.

The fractions of peak D showed about 1000–3000 times lower specific allergenic activity than those of peak C by prick skin testing and by the RAST inhibition, respectively. Parallel inhibition curves indicate either qualitative allergenic similarities between the allergens in these fractions or cross-contamination (1:3000) of peak D fractions with peak C allergens (1, 9).

Our data do not allow a clear distinction of the origin of the allergenically active components of peak D fractions. The amount of peptides was doubled during the long extraction (3–24 h, at 4°C) suggesting that also the active components may be degradation products of the allergens. Birch pollen allergens are sensitive to many proteinases, especially pronase, but relatively resistant to carbohydrases and to some proteinases, e.g.

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