

A Reference Allergen Preparation of the House Dust Mite *D. pteronyssinus*, Produced from Whole Mite Culture – A Part of the DAS 76* Study

Comparison with Allergen Preparations from Other Raw Materials

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A. D. pteronyssinus whole culture allergen preparation contained 49 antigens as revealed by crossed immunoelectrophoresis (CIE), using polyspecific rabbit antibodies. Crossed radio-immunoelectrophoresis (CRIE) with sera from 30 patients revealed nine allergens, antigens 42, X, Y and 23 (in rank order) showing the most frequent and intense IgE-uptake. Nine antigens originated from the culture medium (human dander + yeast), but none of these gave rise to specific IgE-uptake. Extremely few and weak reactions were observed in radioallergosorbent (RAST) with 129 sera, using media extracts on the discs. Purified mite body extract (PMB) contained less ag 42 and more ag Y and ag 23 than whole mite culture extract (WMC), whereas an acetone-extracted mite excreta preparation (AML) contained 5 times more ag 42, but was devoid of ag Y and ag 23. Ag X was present in all preparations. The RAST-inhibitory potency of PMB was best correlated with the content of ag X. Preparations with properties similar to WMC and PMB were judged as suitable for clinical application.

Key words: Allergen; CRIE; DAS 76; *D. pteronyssinus*; RAST.

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CLINICAL ASPECTS

House dust mite allergen preparations can be produced from various raw materials and purification steps may be involved. The present study demonstrates that dialysed extracts of 1) whole mite culture and 2) purified mite bodies are both potent preparations in RAST and both contain allergens important to allergic patients. Both types of preparations are judged as suitable for clinical application, provided non-allergenic food is used for the mite cultivation.

Allergen preparations of house dust mites (genus *Dermatophagoides*) are widely used in allergology for diagnostic and therapeutic purposes (27). However, conditions for cultivation, culture media and further processing (extraction, purification, etc.) differ between labora-

tories. Fractions of the cultivated material (mite bodies, excreta, etc.) as well as the whole culture may serve as the raw material for extraction (10, 22, 24). A variety of different preparations is presently used in clinical practice.

* DAS 76 (Danish Allergen Standardization 1976) is a multicenter study involving the following allergy clinics and laboratories: Allergologisk Laboratorium (ALK), Copenhagen; Frederiksborg Amts Centralsygehus, Hillerød; Esbjerg Sygehus; Københavns Amts Sygehus, Gentofte; Københavns Amts Sygehus, Lyngby; Holbæk Sygehus, Middelfart Sygehus; Nykøbing Falster Sygehus; Proteinlaboratoriet, Copenhagen; Rigshospitalet, Copenhagen; Ringkøbing Sygehus; Thisted Sygehus; Viborg Sygehus.

The present study describes the immunochemical characterization of a *D. pteronyssinus* whole culture allergen preparation (WMC), using immunoelectrophoretic methods (CIE, CRIE and rocket immunoelectrophoresis (RIE)) supplemented by RAST. Special care was taken to control the non-allergenicity of the culture medium and the retention of allergenic activity during extraction and dialysis. Also, a comparison was made between WMC and several other house dust mite preparations, widely differing in manufacture.

An attempt is made to predict the clinical value of these preparations.

MATERIAL AND METHODS

Antigens

Dermatophagoides pteronyssinus allergen preparations were produced from full-grown mite cultures obtained from ALK, Copenhagen. The culture medium was a mixture of human dander from electric shavers and a brewer's yeast product. The mite density was 20–50/mg dry culture. Species specificity was confirmed by microscopical examination of male and female specimens, according to Hughes (7).

Reference mite whole culture extracts (WMC, lot 1–3) were produced from dry mite cultures by 1:10 (w/v) extraction in ammoniumhydrogencarbonate buffer, pH 7.6, $I = 0.125$, containing 0.015 M sodiumazide, at 5°C for 24 h. Subsequent centrifugation (40,000 g, 0°C, 1 h), dialysis (3 × 24 h) and freeze-drying took place as described in (10). CIE showed the three lots to be similar in antigenic composition, and contained the same allergens as shown by CRIE. All quantitative determinations (specific allergen content and RAST-potency) were carried out using lot 3.

Berol solubilization was carried out on lot 1. The pellet obtained during centrifugation was washed 3 times for 1 h at 5°C in water (1 ml/original raw material), containing 1% (v/v) Aprotinin (Novo, Mainz, FRG). Each washing was followed by centrifugation at 27,000 g, 5°C for 10 min. The final pellet was then dissolved

in 0.005 M ammonia containing 2% (v/v) Berol (EMU-043, Modekemi, Stenungssund, Sweden). After stirring for 1 h at 5°C the solution was centrifuged twice at 40,000 g, 0°C for 1 h. The final supernatant with 0.015 M sodiumazide added was stored at –20°C in closed vessels (denoted Berol-solubilized extract).

Small amounts of the centrifugation supernatant (1:10 w/v crude extract) and the dialysed extract from the preparation of the standard freeze-dried extract were stored at –20°C for use as controls.

The yield of freeze-dried material was 48 mg/g raw material, and the protein content was 34% of the freeze-dried weight, as determined by the method of Lowry et al. (15) using bovine albumin as a standard.

Extract of purified mite bodies (PMB) was produced by sieving and salt-flotation as described in (10) and subsequent extraction, dialysis and freeze-drying as above. Extract of mite excreta fraction was analogously produced from the fraction of a mite culture, passing a 45 µm Endecott sieve. Extracts of the culture medium components, human dander and brewer's yeast product, were produced by extraction, dialysis and freeze-drying as above.

The purified allergen preparation (acetone-purified mite leftovers (AML)) was produced from the fraction of a mite culture, passing a 212 µm Endecott sieve. Extraction (1:10 w/v) took place in 44% acetone/56% 0.125 M ammoniumhydrogencarbonate (v/v) for 1 h at 5°C under rotation. The supernatant, after centrifugation at 5000 rpm for 10 min, was mixed 1:1 (v/v) with acetone. The precipitate was washed once in 80% acetone/20% water (v/v) and once in acetone and left for evaporation of residual acetone. After reconstitution in ammoniumhydrogencarbonate buffer (pH 7.6, $I = 0.125$), dialysis and freeze drying was done as above.

The freeze-dried extracts were dissolved in 0.5% (w/v) sodiumchloride, 0.25% (w/v) sodiumhydrogencarbonate/glycerol 50% (v/v) before use in prick test, RAST and immunochemical techniques. Extracts for rabbit immunization were dissolved in 0.1 M sodiumhydrogencarbonate, 0.015 M sodiumazide.

Purified *D. pteronyssinus* major allergen P1 (3) was a gift from Drs. Chapman and Platts-Mills.

Rabbit antibodies. Antibodies to WMC were produced according to (5). The dose of freeze-dried antigen was 1 mg/rabbit/injection. Pooled immunoglobulin fractions from bleedings No. 5–56 of three rabbits were used.

Patient sera. Sera from 71 house dust mite allergic patients originated from the DAS-76 study. Patients selected for the study underwent the following procedure: Patients with a suspected allergy to house dust mite were skin prick tested and, in some cases, subjected to provocation at the participating hospitals. Sera were mailed to the laboratory for RAST. The test extract employed in both cases was the reference WMC (lot 1). Inclusion criteria for patients were: A type 1 allergy for at least 5 years and 2 scores for one test, or at least four scores in all four tests. Score system: Negative = 0, for patients with negative case history, negative provocation test, skin prick wheal size with 0.1 mg/ml extract ≤ 0.5 times the wheal size for histaminehydrochloride 1 mg/ml, RAST-class 0–1. Doubtful = 1, for patients with doubtful case history, doubtful provocation test, wheal size (extract)/wheal size (histamine) < 1 , RAST-class 2. Positive = 2, for patients with positive case history, positive provocation test, wheal size (extract)/wheal size (histamine) ≥ 1 , RAST-class 3–4. Sera from the first 30 of these patients were used for CRIE. Serum-pool from 1000 healthy blood donors was used as normal serum. Serum-pool 1 for RAST-inhibition was a mixture of 12 sera from the DAS-76 study, and serum-pool 2 was a gift from Dr. Annette Ford, NIBSC, London.

Radiolabelled anti-IgE. Anti-human IgE (affinity purified, F_c -specific), labelled with ^{125}I was obtained from Pharmacia AB, Uppsala. The RASTs used for selection of patients, the original CRIE series for identification of allergens in WMC and the control experiments were performed using anti-IgE with specificity for the D_1 and D_2 determinants, while all other experi-

ments used the anti- D_2 antiserum, available from autumn '77.

Rocket-immunoelectrophoresis (RIE) and crossed immunoelectrophoresis (CIE) with modifications (fused rocket immunoelectrophoresis, crossed-line immunoelectrophoresis (CLIE), tandem-CIE, etc.) were performed using the same reagents, gel dimensions and electrophoretic conditions as in (12). A reference immunoplate, stained in Coomassie Brilliant Blue was selected, and a drawing of the precipitation pattern provided with classification numbers. After termination of the CRIE-evaluation, the enumerations were revised so as to include precipitates only visualized through CRIE. Antigens in WMC and other preparations were identified by tandem-CIE, CLIE and antigen-addition experiments. CIE-quantitations of antigen concentrations were performed on 5×5 cm immunoplates without intermediate gel-strip to obtain a well-defined baseline.

Crossed radioimmunoelectrophoresis (CRIE) was performed essentially as described in (26). In short, the dried immunoplates were incubated overnight with 0.5 ml patient serum, control serum or serum pool in 7 ml phosphate-buffered saline, pH 7.0, washed in 0.15 M NaCl and incubated overnight with an amount of labelled anti-IgE, corresponding to 200,000 cpm in our gamma-counter. After washing in 0.15 M NaCl, the plates were dried and placed in X-ray cassettes on X-ray film (Kodak XL 1). Exposure took place at room temperature for successive periods of 1 day, 3 days and 21 days. Precipitates giving rise to radiostaining of the autoradiographs after 1 day of exposure were assigned 10 CRIE-points, appearance after 3 days was assigned 3 points and appearance after 21 days 1 point. These values were reduced by 1 point for precipitates giving rise to radiostaining with the control serum after 21 days of exposure. The evaluation procedure was performed by two persons and their records compared.

Skin-prick test was performed according to (29).

Allergosorbents for RAST and RAST-inhibition were produced from CNBr-activated paper discs (Munktell 00H, diameter 5 mm, 1.8 mg/disc) by coupling 25 discs per ml allergen extract as described in (4). The RAST was performed as described in (18).

Inhibition of serum pool took place overnight at room temperature. Aliquots of 100 μ l serum were added to 100 μ l of each dilution of the allergen preparation to be assayed and to 100 μ l of buffer 1. Normal serum (pool from 1000 blood donors) was added to the most concentrated inhibitor solutions.

The percentage inhibition, $I(x)$, for each inhibitor concentration was calculated according to:

$$I(x) = \frac{\text{cpm}(0\%) - \text{cpm}(x)}{\text{cpm}(0\%) - \text{cpm}(100\%)} \times 100\%$$

where $\text{cpm}(0\%)$ denotes the mean number of counts using buffer as inhibiting solution, $\text{cpm}(100\%)$ denoted the mean number of counts using normal serum instead of pool, and $\text{cpm}(x)$ denotes the mean number of counts using inhibitor solution of concentration $c(x)$.

For statistical analysis of $I(x)$ versus $\log c(x)$ a regression range between 10% and 90% for $I(x)$ was accepted. Each line was compared separately with that of the reference WMC. Requirements for valid assays were: Coefficient of correlation, $r \geq 0.9$ for each regression line, residual variance not statistically different at the 0.01 level under Fisher's v -square distribution. Lines were considered parallel when the slopes were not statistically different at the 0.05 level under the t -distribution.

Control experiments were performed by means of direct RAST and RAST inhibition in order to estimate the allergenic activity of the used standard extract relative to the various subfractions obtained during the preparation procedure, and, also, to control the success in raising rabbit antibodies against all extracted allergenic components.

Cyanogen bromide-activated paper discs were coupled with the following extracts: Crude extract (1:10 w/v), Berol solubilized extract and the standard freeze-dried extract (WMC).

Serum pool 1 (used undiluted or diluted 1 + 9) was inhibited with 1) crude extract (1:10 w/v), 2) standard extract, 3) the RAST-buffer, or 4) Berol-solubilized extract. RAST-inhibition was carried out as described above.

Success in raising rabbit antibodies against the allergens included in the extract was controlled by immunoabsorption of the extract with immobilized rabbit antibodies to WMC. Immobilized antibodies to *Avena elatior* grass pollen extract serves as a control. Coupling of rabbit antibodies (1 ml) to 1 g dry cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala) was carried out according to the manufacturer's instructions. The immunoabsorbents were equilibrated with sodium citrate buffer (pH 6.0, $I = 0.05$) and finally prepared as small 2 ml columns before use. To each column was applied 0.24 mg WMC plus 0.1 mg human serum albumin (Kabi, Stockholm) diluted in 50 μ l sodium citrate buffer. This corresponds to a five-fold excess of immobilized antibody on the column relative to the CIE plate. The columns were eluted with 6 aliquots of 0.5 ml of sodium citrate buffer (pH 6.0, $I = 0.05$) followed by 6 aliquots of 0.5 ml sodium citrate buffer (pH 6.0, $I = 0.5$). The 12 fractions were submitted to fused rocket immunoelectrophoresis against rabbit anti human serum albumin (DAKOpatts, Copenhagen). The fractions containing human albumin (used as a marker for protein elution) were pooled, and, after addition of 200 μ l 1.0 M sodium hydrogen carbonate, used in direct RAST.

RESULTS

Antigenic composition of WMC

A total of 49 precipitates appeared on the CIE precipitation pattern for the reference whole culture extract (Dp-WMC) (Fig. 1). Two further precipitates were identified through their appearance on the CRIE-autoradiographs and were designated X and Y, respectively. According to the positions of the corresponding precipitates, antigens 1-43 and X are an-

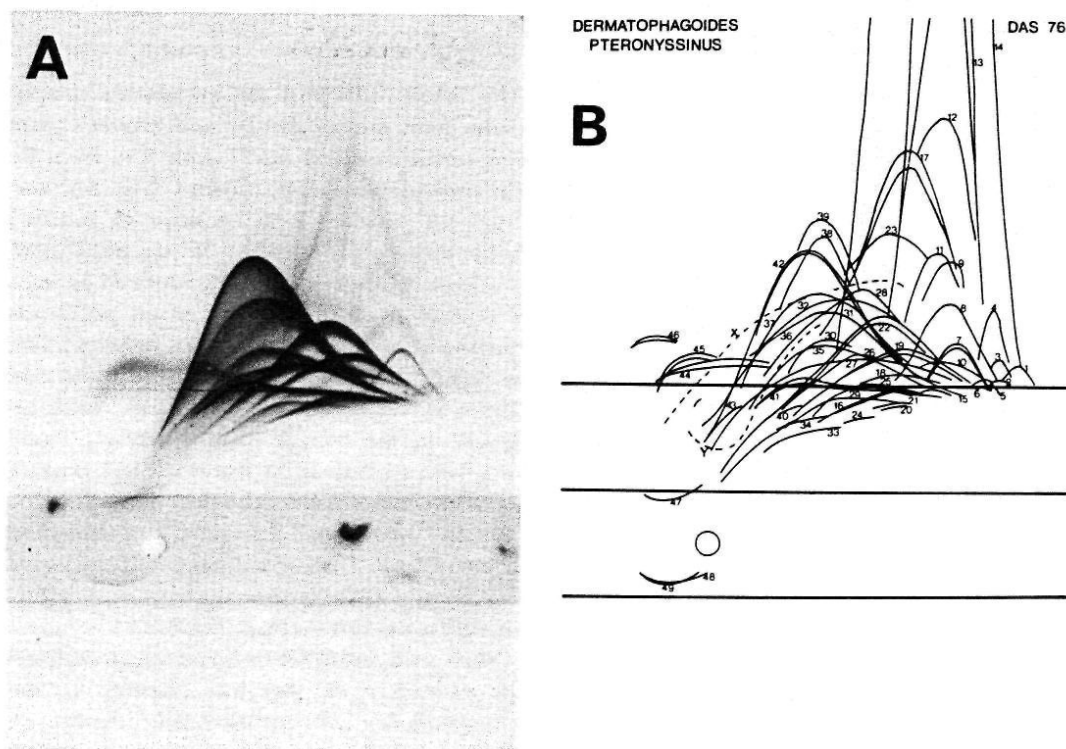


Fig. 1. A: Crossed immunoelectrophoresis (CIE) of whole mite culture extract (WMC), 100 μ g applied in well. Anodical antibody-containing gel with 3.4 μ l/cm², cathodical gel with 3.3 μ l/cm². A: 1 cm broad intermediate gel without antibody was placed on the anodical side of the 1st dimension gel. Agarose: Litex, type HSA. Electrophoretic conditions: 1) dimension 10 V/cm for 30 min. 2) dimension: 2 V/cm for 16 h. Plate pressed, washed, dried and stained with Coomassie Brilliant Blue. B: Reference drawing of A. Precipitates enumerated arbitrarily from right to left, according to average migration velocity. Precipitates X and Y (only visible after CRIE) indicated with stipulated lines.

odically migrating, antigens 47–49 and Y are cathodically migrating, and antigens 44–46 of near-zero mobility. The position of the latter in the upper antibody-containing gel is probably caused by the dilution of agarose with antibody solution and, consequently, a lower electro-osmotic force operating in the second dimension electrophoresis.

Crossed line immunoelectrophoresis (CLIE) with human dander extract in the intermediate gel demonstrated precipitates 8, 9, 11, 13, 19, 22, 35, 47 and 49 to contain human components, originating from the culture medium. Only reactions of total identity were observed. Total absence of identity reactions was observed with the yeast extract in CLIE.

Control experiments

The standard extract was able to inhibit the RAST-reaction to discs coupled with the crude (1:10 w/v) extract and the Berol solubilized residue after extraction (Fig. 2). The inhibition was roughly equal to that obtained with the coupling extracts themselves, as well as to that with standard extract inhibiting the RAST-reaction to discs with standard extract.

The passage of the standard extract through a column with immobilized antibodies to WMC led to a complete removal of RAST activity from the extract (Fig. 2B). The radioactivity retained on discs coupled with absorbed extract equalled that on discs coupled with HSA alone.

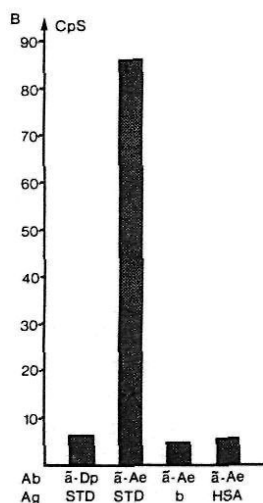
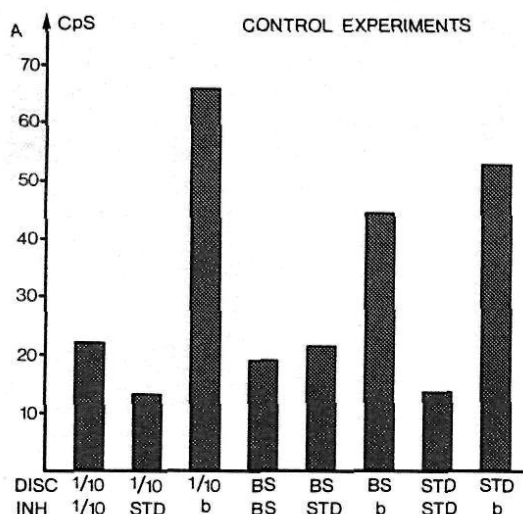


Fig. 2. Result of RAST experiments performed to assess the allergenic activity of the standard extract (WMC) and the quality of the rabbit anti-WMC antibodies. A: RAST inhibition experiments. "Disc" and "INH" indicate the various allergosorbents and inhibitors used. 1/10: crude 1:10 (w/v) extract; BS: berol-solubilized material; STD: standard WMC; b: incubation buffer. B: Column absorption followed by direct RAST. Ab: immobilized antibodies used; a-Dp: anti-Dp-WMC; a-Ae: anti-*Avena elatior* pollen; Ag: preparation passed through columns. HSA: human serum albumin. RASTs performed with serum pool 1. CpS: retained radioactivity in counts per second.

Allergenic activity of culture medium components

The RAST-activity of the extractable components from human dander and brewer's yeast was tested by direct RAST with sera from 71 *Dermatophagoides*-allergic patients (Fig. 3A) and from 28 patients, RAST-positive to purified house dust extract (Fig. 3B). No reactions above the level of unspecific binding (defined as twice the reaction of the normal serum pool) was observed for the yeast extract, whereas one serum in the *Dermatophagoides* group and one serum in the house dust-group gave weak reactions to the human dander extract. Three sera from a group of 30 patients, RAST-positive (class 3–4) to various moulds (including the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Mucor*, *Phoma*, *Trichoderma* and *Ulocladium*) gave a moderate reaction (less than 10% of added counts) to the yeast extract (Fig. 3C).

Skin prick test in seven *Dermatophagoides*-allergic patients gave negligible reactions to the human dander and yeast extracts (Table 1).

Identification of allergens in WMC

CRIE demonstrated IgE/anti-IgE uptake from 13 precipitates as shown by visible radiostaining on the X-ray film after 21 days of exposure (Table 2). The control serum pool demonstrated radiostaining from six precipitates after 21 days (16, 17, 19, 22, 41 and 42), but the 3-day autoradiograph was totally negative. Thus radiostaining from these six precipitates after 21 days was considered due to unspecific binding of IgE/anti-IgE. Only precipitate 42 gave rise to radiostaining after 1 day of exposure. Examples of autoradiographs are shown in Fig. 4. Seventeen out of 30 patients' sera showed specific radiostaining in CRIE. Antigens 42, X, Y and 23 were the antigens most frequently binding specific IgE (15, 12, 10 and 10 sera, respectively). None of the specifically IgE-binding antigens originated from the culture medium.

Immunochemical identity of Dp 42 and P₁

When 4 µg antigen P₁ was investigated in CIE, one major precipitate and a few weak precipi-

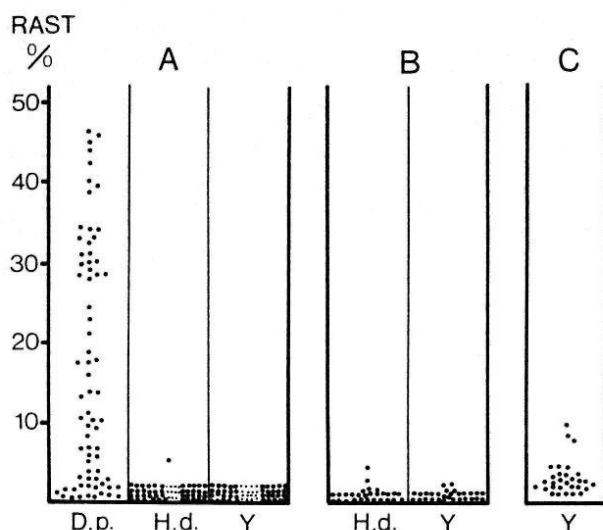


Fig. 3. RAST-response to culture medium components. *D. pteronyssinus* WMC, human dander extract (H.d.) and yeast product extract (Y) all coupled at concentrations 1 mg/ml. Figures on ordinate denote the retained radioactivity as percent of added activity. A: 71 patients, allergic to *Dermatophagoides*. B: 28 patients, RAST-positive to house dust extract. C: 30 patients, RAST-positive to various moulds (see text).

tates (near the base line) were observed. CRIE with patient serum pool 2 demonstrated a powerful radiostaining of the former precipitate after 3 days autoradiography. No significant radiostaining was observed for the minor precipitates, which were accordingly interpreted as contaminants. Tandem-CIE demonstrated total immunochemical identity between ag 42 of WMC and antigen P_1 .

Antigenic/allergenic composition of AML and PMB

Important allergenic components (ags 42, X, Y and 23) were identified by tandem-CIE and CLIE. Both AML and PMB contained ags 42 and X as shown by CIE and CRIE (Fig. 5). Ags Y and 23 were further identified in PMB, but not in AML. Quantitations performed by CIE demonstrated that, compared with WMC, AML

Table 1
Skin prick test reactions (mm wheal diameter) to extracts of mite culture and medium

Patient No.	Positive control ¹	Negative control ²	Mite culture (0.3 mg/ml)	Human dander (0.1 mg/ml)	Yeast product (0.1 mg/ml)
1	4.5 × 4.5	0 × 0	10.0 × 5.5	0 × 0	0 × 0
2	5.5 × 4.5	0 × 0	2.5 × 2.5	0 × 0	0 × 0
3	5.5 × 4.5	0 × 0	6.0 × 4.0	0 × 0	0 × 0
4	4.0 × 4.0	0 × 0	6.0 × 5.0	0 × 0	0 × 0
5	4.0 × 4.0	0 × 0	8.0 × 5.0	2 × 1	0 × 0
6	5.0 × 3.5	0 × 0	6.5 × 4.0	2 × 2	0 × 0
7	8.0 × 7.0	3 × 2	8.0 × 5.0	3 × 2.5	3 × 2

¹ Histamine-HCl, 1 mg/ml.

² Solution fluid, saline/glycerol (50%/50%).

Table 2
CRIE-response from individual patients to antigens of *D. pteronyssinus*

Patient ¹	RAST- class	Skin-Index at 1 HEP	Σ(CRIE)	CRIE-points for antigens												
				14	16	17	19	22	23	29	30	37	41	42	X	Y
1/45	4	1.71	18			2	0	0	3	1	3		0	9		
2/5	4	1.40	11		0			0	3					2	3	3
2/27	4	1.16	11		0	0	0	0			1		0	2	3	3
2/34	4	0.87	17				0	0	1		1		0	9	3	3
3/2	4	1.85	10				0	0	1		1			2	3	3
3/16	4	0.67	16	3			0	0	3		1	1	0	2	3	3
3/19	4	0.71	22		0		0		3		1	3	0	9	3	3
3/32	4	1.10	7				0	0	1				0	2	3	1
3/33	4	0.98	6			0	0						0	2	3	1
1/8	3	—	1									1		0		
2/8	3	1.14	7		0		0	0	1				0	2	3	1
2/14	3	1.00	4			0	0	0						2	1	1
3/31	3	0.66	3				0	0			0		0	2		
1/1	2	—	3				0		1				0	2		
1/10	2	1.92	4				0	0				1	0	2	1	
3/40	2	0.98	3	3			0	0					0	0	3	
3/3	0-1	0.51	2				0	0					0	2		
1/41	3	0.38	0											0		
1/43	2	—	0				0						0	0		
2/7	2	0.88	0											0		
3/30	2	0.50	0		0		0	0						0		
1/3	0-1	0.52	0				0	0					0	0		
3/8	0-1	—	0											0		
2/1	3	—							+							
2/18	3	1.17										+		+	+	+
2/23	3	1.87										+		+	+	+
1/32	2	0.46														
1/33	2	—														
2/17	2	—														
2/24	2	0.52													+	

¹ Top section: patients with positive CRIE-response after correction for unspecific binding. Middle section: patients with negative CRIE after correction. Bottom section: Sera totally CRIE-negative; positive radiostaining after 3 days at retest stated with +.

Table 3

CIE-determination of allergen content in WMC, PMB and AML: (percentage relative to WMC (mg vs. mg))

	Ag 42	Ag X	Ag Y	Ag 23
WMC	100	100	100	100
PMB	35	170	200	>200
AML	470	125	0	0

is enriched approx. 5-fold in ag 42, whereas PMB contained relatively small amounts of ag 42 and relatively large amounts of ag Y and ag 23 per dry weight (Table 3):

There was 10-times more ag PMB-2 in PMB than in WMC (Fig. 5). In contrast to WMC, only few culture medium components (human dander proteins) could be identified in AML

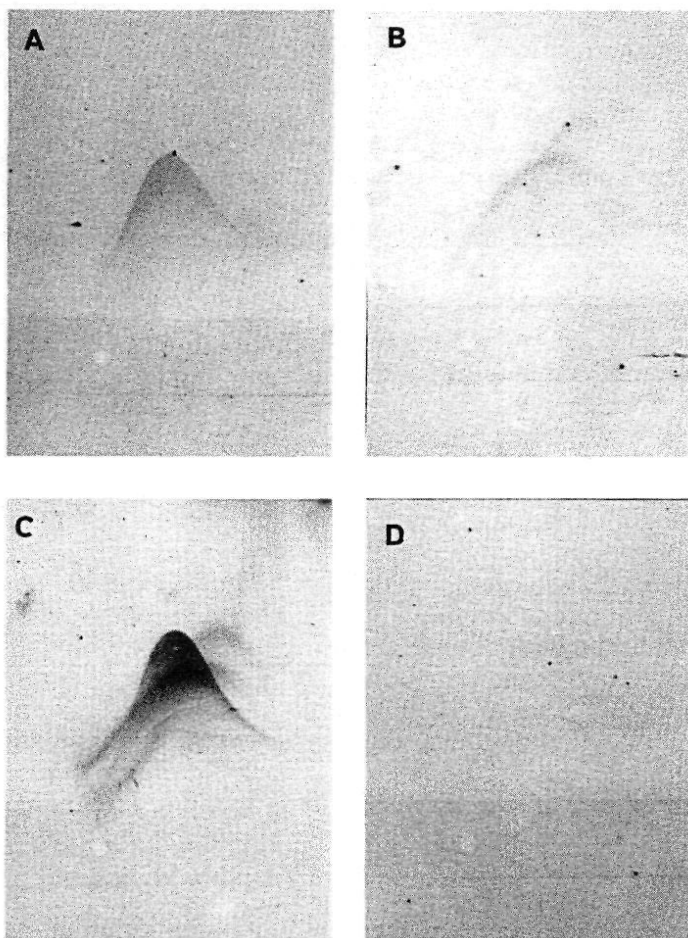


Fig. 4. Examples of CRIE-autoradiographs after 21 days of exposure. A: Patient 3/3; specific response to ag 42. B: Patient 3/23; specific response to Ags X and 23. C: Patient 3/16; specific response to Ags 42, X, Y, 23, 14, 30 and 37. D: Control serum pool (from 1000 blood donors).

and PMB by CLIE. These appeared as weak precipitates near the base line.

Ag 37, identified as a minor allergen in the DAS-76 study (Fig. 4) gave rise to strong IgE-uptake in CRIE with the IgE-serum pool used in this case.

RAST-activity of AML and PMB

Using WMC as a standard preparation in RAST-inhibition, AML and PMB had the capacity to inhibit the RAST-reaction completely (Table 4). The slope of the regression line on the % inhibition versus log inhibitor concen-

tration was, however, statistically different ($P < 0.001$) between AML and WMC, prohibiting calculation of relative potency. The calculated RAST-inhibitory potency of PMB was 280% relative to WMC.

The RAST-activities of WMC, AML and PMB, using individual patient sera, were compared in two separate experiments. In the first experiment, 70 sera from the DAS-76 study were used to correlate reactions to WMC, PMB and mite excreta extract (Fig. 6A–B). In the second experiment, 24 sera from the same population were used to compare reactions to mite excreta extract and AML (Fig. 6C). An

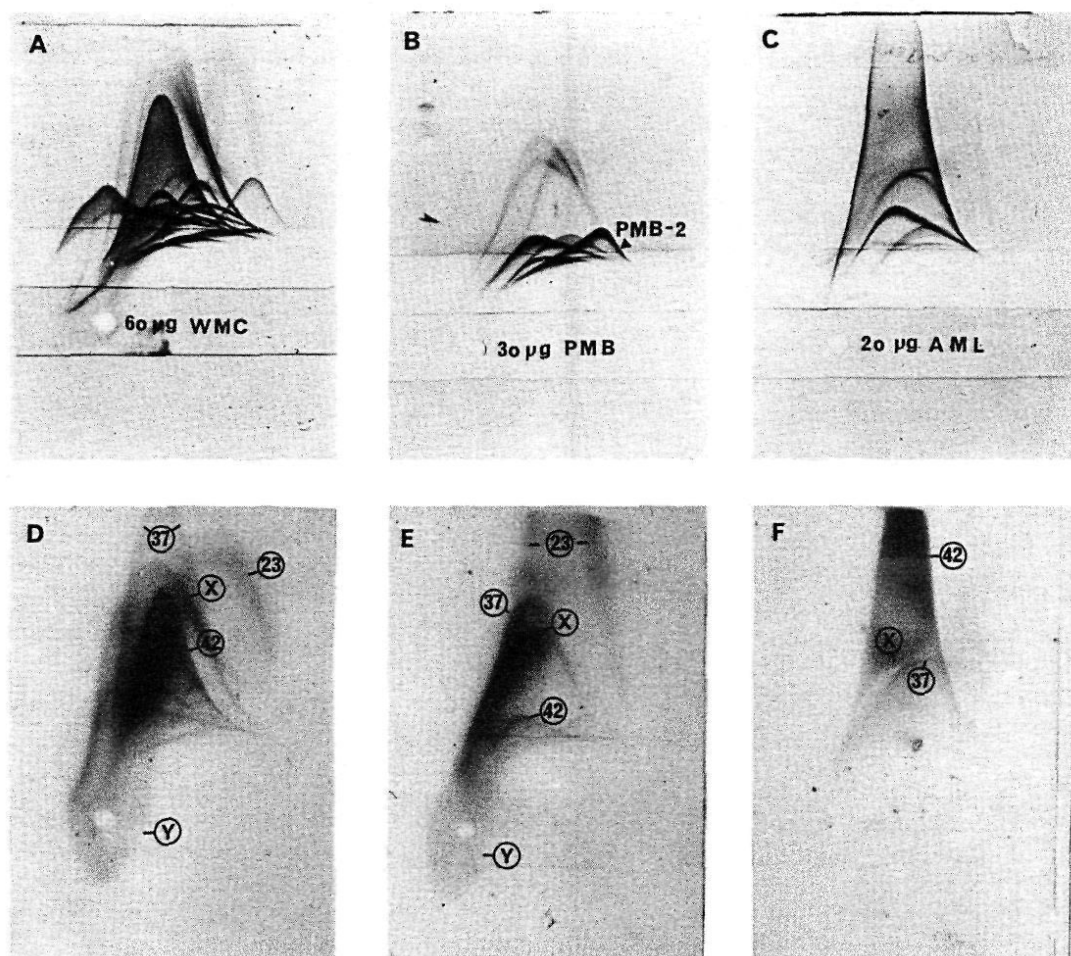


Fig. 5. Allergenic composition of WMC, PMB and AML. A – C: Crossed immunoelectrophoresis, employing $3.4 \mu\text{l}/\text{cm}^2$ anti-WMC in anodical gel and $3.3 \mu\text{l}/\text{cm}^2$ in the cathodical gel. D – F: Corresponding crossed radioimmuno-electrophoresis, employing patient serum pool 2.

Table 4
RAST-inhibition assay of WMC, PMB and AML.

	Maximal inhibition	Regression range (% I)	Correlation coefficient	Slope I/logC	C (50 % INH)	Parallelism (with WMC)	Relative potency
WMC	100	12–84	0.99	37	–3.0	–	100
PMB	100	11–76	0.93	26	–3.5	yes ($P > 0.1$)	280
AML	100	27–76	0.99	19	–2.7	no ($P < 0.001$)	–

Discs coupled with WMC, 3 mg/ml.
Patient serum pool 2, diluted 1 + 3.

almost perfect linear correlation ($r = 0.99$) was obtained between WMC and mite excreta extract. A good correlation ($r = 0.95$) was likewise obtained between WMC and PMB. Two patients, however, reacted with a 3–4 fold larger IgE-binding to PMB. The RAST-reactions to AML and mite excreta extract also correlated well ($r = 0.94$), only one patient reacting with significantly less RAST-binding to AML.

An overall equivalence of the direct RAST-activities of WMC, PMB and AML was thus observed.

DISCUSSION

The purpose of this investigation was to establish a classification of the allergenic constituents of *Dermatophagoides pteronyssinus*, as complete as possible, and to use this as a framework for evaluating the potential usefulness of various mite allergen preparations in clinical practice and the laboratory. For this purpose a reference extract was produced.

Two important choices had to be made: 1) the proper raw material, and 2) a proper process of manufacture.

Although there is convincing evidence of a specific major sensitization to mite faecal particles (22), the absence of sensitization to other mite materials (e.g. glandular contents, and dried-up body fluid from disintegrated mites) has not been excluded. However, we postulate that the fully grown mite culture, with its content of mite bodies and excrements/secretions, corresponds to the products of the growing mite population in the patients' bed and bedroom. Thus, we used whole mite culture as the raw material for the reference extract.

Extraction was carried out under mild physico-chemical conditions imitating those prevailing in the respiratory mucosa. To reduce degradation caused by microbial growth and possible proteolytic enzymes, temperature was kept low and preservative added. No potentially denaturing agents, such as phenol or organic solvents were used. In order to im-

prove stability and remove low-molecular weight (<8000) potential irritants in skin test and other provocation tests, the extract was dialysed and freeze-dried.

Polyspecific rabbit antibodies were raised against this preparation (WMC), using long-term immunization, known to favour a broad spectrum of antibody specificities with good precipitating power.

The assumptions of: 1) The presence in the reference extract of the complete repertoire of allergens in the raw material and 2) the presence in the rabbit antibodies of corresponding specificities, which together constitute the WMC/anti-WMC reference, system, were tested by RAST-experiments. The reference extract was shown to inhibit the RAST-reaction to a crude 1:10 (w/v) extract (before dialysis) and Berol, a preparation produced by solubilizing the extraction residue in the non-ionic detergent. The rabbit antibodies were further shown to absorb all RAST-activity from the reference extract, as shown by column absorption and subsequent RAST.

This is no proof of the completeness of the reference system, as the absence of certain allergens may not be detected in the RAST, e.g. due to lack of IgE specificities in the patient serum pool or due to lack in certain allergens of accessible amino groups for the covalent coupling to CNBr-activated cellulose. However, we consider the existence of a major IgE-binding allergen, detectable in neither RAST nor CRIE, to be unlikely, as the molecular requirements for covalent coupling to sepharose and antigenicity towards rabbit antibodies are quite unrelated.

It is important to test for a possible influence from extractable components in the culture medium, as the eventual presence of allergens from this source may seriously limit the value of the extract in diagnosis and immunotherapy and give rise to erroneous interpretations of laboratory data, obtained by e.g. RAST. Mites can be cultivated on a wide variety of media, including dried *Daphnia* (16), dog food (20) and skin scales (dander) from man (23) or animals (19), usually in mixture with yeast products. Some of these media contain powerful allergens.

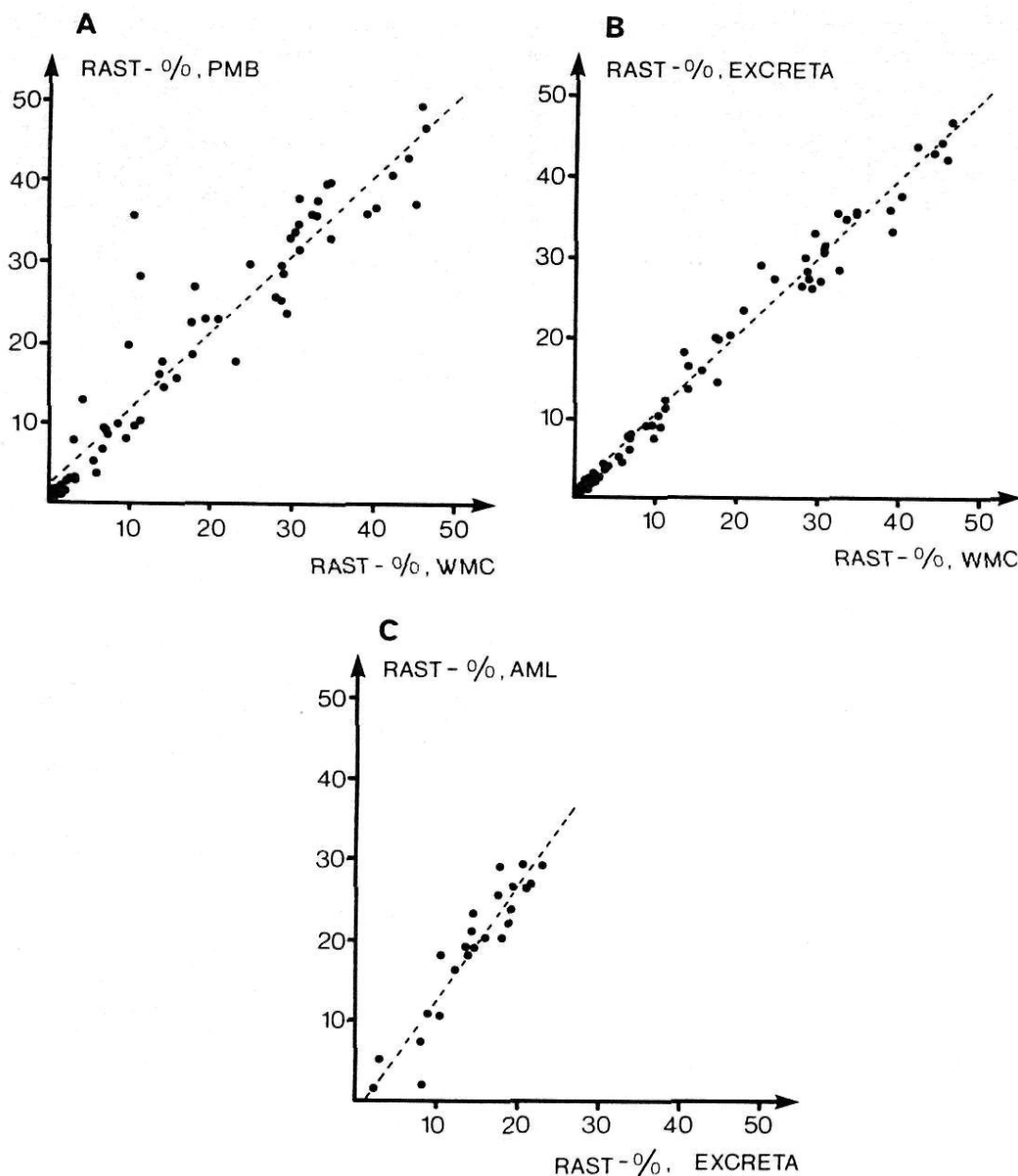


Fig. 6. Direct RAST-activities of WMC, PMB and AML. Figures on axes denote the retained radioactivity as percent of total added activity. Coupling concentration in all cases: 1 mg freeze-dried extract/ml. A: 71 patients' sera (coefficient of correlation, $r = 0.95$). B: 71 patients' sera ($r = 0.99$). C: 24 sera ($r = 0.94$).

Some controversy exists regarding the allergenicity of the human dander and yeast mixture, originally prescribed for mite cultivation (24). Positive RAST's to human dander (2) and

to yeast (20) have been reported as have opposite findings (6, 18).

Mite cultures employed by us were grown on a mixture of human dander and a brewer's

yeast product. RAST-reactions to these medium components were weak and infrequent (Fig. 3), with the possible exception of sera from patients allergic to moulds, as 3/30 patients' sera apparently contained IgE, directed against the yeast extract. This probably presents no obstacle to the clinical use of mite extracts cultivated on this material, as a considerable part of the medium is digested by the mites in fully grown cultures. Remaining yeast components appear only weakly immunogenic, as no rabbit antibody response was elicited by immunization with the whole culture extracts. However, this does not exclude the possibility that specific IgE/IgG to these antigens could be induced in patients undergoing immunotherapy.

The response of patients' sera in CRIE followed a pattern analogous to that revealed in most other allergen extracts such as dander from dog (1), horse (13) and cow (17), and pollen from timothy (25) and ragweed (14): one or a few major allergens, to which almost any CRIE-positive patient serum responds, and several intermediate/minor allergens with varying frequency of specific IgE-binding. The specificity of the binding in CRIE is most clearly demonstrated by the different response patterns to the intermediate/minor allergens, e.g. allergens 23 and Y (Table 2).

Thirteen sera gave rise to no specific response in CRIE. This may be a reflection of the rather permissive inclusion criteria used. Some patients have been included on the basis of a positive anamnesis or a positive skin prick test alone and others on the basis of a doubtful result in all performed tests. By this design it was intended to minimize rejection of patients

with possible restrictions of IgE-specificity to minor allergenic components.

Six of the CRIE-negative sera responded to at least ag 42 after 21 days of exposure. Such reactions were considered unspecific, because the control sera (from 1000 healthy blood donors), were also positive at this exposure time. It is generally assumed that this is caused by unspecific uptake of IgE/labelled anti-IgE to dense precipitates (as in this case Dp-42). It must, however, be anticipated that there may be a low level of specific IgE to *Dermatophagoides* in this control pool due to the frequent occurrence of sensitization to house dust mite. We estimate a 2% contribution from mite allergic patients to this normal serum pool. Low levels of specific IgE may, furthermore, be present in individuals exposed to house dust mites but presenting no clinical symptoms. The remaining seven sera, showing no CRIE response at all, were retested in CRIE to both WMC and PMB. Specific IgE-binding was observed for four of these sera (2/1, 2/18, 2/23 and 2/24) after 3 days' exposure. No difference was observed in occurrence or intensity of radiostaining to WMC and PMB. As a technical error in the first CRIE-study probably gave, in some cases, falsely negative results, no distinct classification in major, intermediate and minor allergens was attempted. That ags 42, X, Y and 23 are the most important allergens, and in the stated rank order, is justified on the basis of the positive CRIE-autoradiographs.

In a recent CRIE-study (10), using extract of mite-bodies (PMB) and patient sera selected according to the same criteria as in the present study, antigens identical to 42 and X were iden-

Table 5
Identities of *D. pteronyssinus* allergens

Preparations investigated		Allergen enumerations					Reference
WMC	42	X	Y	23	37		Present study
PMB	20	27	26	10	18		(10)
WMC, PMB, WMC	IV	II	V	I	III		(11)
Purified material	P ₁	—	—	—	—		(3)

tified as major allergens, whereas Y and 23 were identified as intermediate allergens (Table 5).

Antigen 42 is immunochemically identical to the major allergen, P1, (3), the importance of which was assessed by radioimmunoassay and skin test.

Preparation of mite extract by extraction of the fully grown, whole mite culture was the procedure originally established by Voorhoorst and colleagues (23) and still advocated by them (24). Potent mite preparations may, however, be produced from the mite bodies themselves after separation from medium and excrement particles (10, 16, 21) or by purification procedures, using organic solvent precipitation (8, 9). Such procedures may serve to remove the bulk of culture medium components, and increase allergenic activity, relative to total macromolecular weight.

The mite body extract (PMB) produced in our laboratory contained relatively small amounts of the major allergen, ag 42, but relatively large amounts of the intermediate allergens, ag X, Y and 23 (Table 3) and was of larger RAST-inhibition potency than the whole culture extract, when compared on a weight to weight basis (Table 4).

A strong correlation existed between the RAST-response of individual sera to WMC and PMB. Two patients (3%), however, reacted significantly stronger to PMB, indicating the existence of minor allergens of somatic origin, present in PMB due to the maceration step in the extraction of the mite bodies.

The organic solvent purified extract, AML, was deficient in the allergens Y and 23, as shown by CRIE. This was not directly reflected in the RAST-data, as AML was able to inhibit completely the RAST-reaction to WMC-discs. Furthermore, almost identical results in RAST with individual patients' sera was produced with mite excreta extract and AML. This probably reflects a rather low sensitivity of RAST-assays in detecting absence of single allergens from allergen extracts.

Another aspect of these data is the apparent lack of relation between RAST-inhibitory potency and content of Dp-42, when comparing WMC, PMB and AML. In fact, an almost in-

verse relationship was found, with concentrations giving 50% inhibition of RAST. It has been a general observation in our laboratory that RAST-inhibitory potency correlates better with the concentration of the second major allergen, Dp-X. This allergen has now been produced with reasonable purity, and studies of its properties are in progress.

The information obtained by immunoelectrophoretic methods – CIE, CRIE, RIE and modifications – furnish, together with RAST-data, a detailed characterization of mite extracts on the molecular level. Such information may serve as a background for the selection of international reference preparations and for comparative studies of clinical efficacy.

The purified preparation, AML, was superior to the other two regarding concentration of the major-allergen, Dp-42, but deficient regarding the intermediate allergens Dp-Y and Dp-23. A general therapeutic use of preparations of this kind would probably leave a large fraction of the patients without sufficient immunostimulation with these allergens. However, provided a sophisticated diagnostic evaluation is made, using e.g. CRIE, patients with their specific IgE response restricted to Dp-42 and Dp-X, could probably be effectively treated with AML.

Whole mite culture extract (WMC) and purified mite body extract (PMB) appeared to be comparably potent, and both contained the full repertoire of important allergens. However, these were present in different relative concentrations and mixed with different irrelevant antigens. Although both types of preparations are probably well suited for clinical application, we should warn against substituting them for one another during the clinical investigation and treatment of the patient. Information from the manufacturers of allergen preparations as to which type of raw material has been used should be easily accessible. Both types of preparations are in common use in clinical practice, but a direct comparison of their clinical efficacy has yet to be made.

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