

Demonstration of Close Physicochemical Similarity and Partial Immunochemical Identity between the Major Allergen, Dp42, of the House Dust Mite, *D. pteronyssinus* and Corresponding Antigens of *D. farinae* (Df6) and *D. microceras* (Dm6)

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Abstract. House dust mite antigens Df6 and Dm6, corresponding to *Dermatophagoides pteronyssinus* (Dp) antigen 42 (= P1), were purified from cultures of *Dermatophagoides farinae* (Df) and *Dermatophagoides microceras* (Dm) using hydrophobic interaction chromatography in combination with gel filtration or acetone precipitation. Purified antigens from the three mite species (Dp42, Df6 and Dm6) were similar regarding molecular weight by gel filtration (*D. pteronyssinus*: 17,000, *D. farinae*: 17,000 and *D. microceras*: 18,500) and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (*D. pteronyssinus*: 30,000, *D. farinae*: 28,000 and *D. microceras*: 29,000). Widely spaced isoelectric points were observed for the antigens of all three species by sucrose gradient isoelectric focusing: (*D. pteronyssinus*: 4.5 and 6.3, *D. farinae*: 4.9 and 6.9 and *D. microceras*: 4.9 and 6.5). Species-specific rabbit antibodies were easily detected for all three species in crossed-line immunoelectrophoresis using mixtures of the two heterologous antigens in the intermediate gel. Antibody specificity for an epitope, common to *D. farinae* and *D. microceras*, but not shared by *D. pteronyssinus*, was further demonstrated. Potent rabbit antibody reagents with species specificity were produced by column immunoabsorption. Despite a probable common molecular structure and origin, the antigens of the three species exhibited gross differences in their immunochemical reactions with rabbit antibodies.

Introduction

The allergenic importance of house dust mites of the genus *Dermatophagoides* is well-documented [15, 16]. However, relatively scarce information is available about the degree of allergenic identity between the *Dermatophagoides* species. Based on skin test results, Voorhoorst and Spijkema [14] concluded that *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* produce the same allergen. A large degree of equivalence between the two species was also obtained in a recent study involving skin-prick tests and RAST [12]. Common antigens in extracts of the two species have been demonstrated by immunodiffusion [1, 4].

The recent progress in identification and purification of house dust mite allergens [3, 7, 9] now allows a more detailed comparison of the corresponding major allergens of the species. The results presented here demonstrate striking immunochemical differences between the corresponding antigens of the three in-

vestigated species (Dp42 (= P1), Df6 and Dm6) despite large similarities in physicochemical properties.

Material and Methods

Antigens

Cultures of *D. pteronyssinus*, *D. farinae* and *D. microceras* were maintained at ALK, Copenhagen, using human dander and a dried brewers yeast product as food supply. Species identity and homogeneity was assured by microscopical examination using a Leitz transmission microscope allowing 1,250 $\times$ magnification. Hughes [6] was used as identification key. The size and shape of the bursa copulatrix, the position of the female genital setae and the size of the spine on tarsi I and II were used as the discriminating characters between *D. farinae* and *D. microceras* [5]. Samples of *D. farinae* and *D. microceras* cultures were kindly examined by Dr. A. Cunningham, Pest Infestation Control Laboratory, Slough, UK who confirmed species identity and homogeneity.

Reference whole mite culture extracts (Dp-WMC, Df-WMC and Dm-WMC) were produced by cold extraction in ammonium hydrogencarbonate buffer, pH 7.6, centrifugation, dialysis and freeze-drying as previously described [9]. Characterization and

Antigens Dp42, Df6 and Dm6

identification of allergens in Df-WMC and Dm-WMC will be the subject of a separate publication.

Acetone-purified excrement preparations (Dp-AML, Df-AML and Dm-AML) were produced by extraction in 44% acetone/56% ammonium hydrogen carbonate buffer (v/v) and precipitation in 80% acetone/20% water (v/v), dialysis and freeze-drying [9].

Purification of the major allergen of *D. pteronyssinus*, Dp42, has been described elsewhere [7]. The corresponding allergen of *D. farinae* (Df6) was produced from Df-AML by essentially the same procedure: a 10-ml phenyl-Sepharose CL-4B column was equilibrated in 0.05 M sodium acetate buffer, pH 5 (AB), containing 1.0 M ammonium sulfate, and 30 mg Df-AML was applied, dissolved in the equilibration buffer. 5-ml fractions were eluted with a flow rate of 15 ml/h and the following buffers: (1) 20 ml equilibration buffer, (2) 20 ml AB + 0.2 M ammonium sulfate, (3) 20 ml AB, (4) 20 ml 0.05 M phosphate buffer, pH 7.0, (5) 20 ml 0.05 M sodium carbonate buffer, pH 10.0 and (6) 40 ml 50% carbonate buffer, pH 10/50% ethyleneglycol (v/v). Fractions 24-27, containing the main part of Df6 and minimal amounts of contaminants as revealed by fused-rocket immunoelectrophoresis (FRIE) were pooled, dialyzed against 0.005 M ammonium hydrogen carbonate and freeze-dried.

Allergen Dm6 of *D. microceras* was produced by Sephadex G-75 gel filtration of 490 mg Dm-WMC (reconstituted in 8 ml 0.125 M ammonium hydrogen carbonate) on a Pharmacia K 26/100 column (bed volume 480 ml, flow rate 11.2 ml/h, 2.7 ml/fraction). Fractions 123-140, with apparent molecular weights (MW) between 12,000 and 16,000, were pooled, dialyzed against 0.05 M sodium acetate buffer, pH 5.0, containing 1 M ammonium sulfate, and applied to a 10-ml phenyl-Sepharose CL-4B column equilibrated in the same buffer. Elution took place as described for Df6 above. Fractions eluted with ethyleneglycol/carbonate buffer, pH 10, shown by FRIE to contain the majority of Dm6 and a minimum of contaminants, were pooled, dialyzed and freeze-dried.

The yield of material was small - approximately 1 mg in both cases - reflecting recoveries of 3-4% of the specific allergen content in the starting materials. The purification ratios (relative to reference WMC extracts) appear in table I. Difficulty to obtain a reliable dry weight for Df6 allowed only an estimation based on the protein weight of the purified component.

Rabbit Antibodies

Polyspecific antibodies to Dp-WMC and monospecific antibodies to Dp42 were the same as previously applied [9], and polyspecific antibodies to Df-WMC and Dm-WMC were each produced in 2 rabbits using the same immunization schedule and with doses of 0.75 and 1.0 mg extract/rabbit/injection. Monospecific antibodies to Df6 and Dm6 were produced with doses of 6 and 25 μ g antigen/rabbit/injection.

Immunoelectrophoresis Techniques

Rocket immunoelectrophoresis (RIE), crossed immunoelectrophoresis (CIE) and variations thereof [crossed-line immunoelectrophoresis (CLIE), FRIE] were carried out as described in [11].

Isoelectric Focusing

Sucrose gradient isoelectric focusing was carried out on 20 mg Dp-AML, Df-AML and Dm-AML as previously described [7] using a 140-ml LKB 8101 column and a voltage of 200 V (2.2 mA) for 48 h. 2.5-ml fractions were collected, the pH of each fraction measured and FRIE performed.

Table I. Purification of Df6 and Dm6

	Purification procedure	Purification effect I ¹	Purification effect II ²
Df6	AML-phenyl-Sepharose	not determined	11.6
Dm6	WMC-G-75 gel filtration phenyl-Sepharose	10.6	14.5

¹ Calculated as $\frac{\mu\text{g WMC applied}}{\mu\text{g purified antigen applied}}$ at the same rocket height in RIE.

² Calculated as above, but using protein weight of purified component (determined by the Lowry method with bovine serum albumin as the reference).

Gel Filtration

Experiments for determination of MW were performed on 485 ml Sephadex G-75 packed onto a Pharmacia K26/100 column, equilibrated in 0.125 M ammonium hydrogen carbonate. 20 mg AML of each species was dissolved in 1 ml equilibration buffer, applied to the column and eluted with equilibration buffer using a flow rate of 11.2 ml/h. A parallel experiment was performed with the following MW markers: human serum albumin (68,000), ovalbumin (45,000), chymotrypsinogen (23,000), myoglobin (17,000) and cytochrome c (12,000). Based on the result of FRIE, fractions 60-86 (Dp and Df) and fractions 53-80 (Dm) were investigated in RIE using monospecific antibodies, and the apparent MW calculated from the fraction producing maximum rocket height using the standard plot of logMW against Kav.

Sodium Dodecyl Sulfate Polyacrylamide Gel (SDS-PAGE) Electrophoresis

PAGE electrophoresis in the presence of SDS was carried out as a modified Laemmli procedure as described in [13].

Column Absorption

Small 5-ml plastic syringe columns were prepared with 20 mg Dm-AML and 50 mg Dp-AML coupled onto 0.5 and 1.0 g CNBr-activated Sepharose 4B (Pharmacia, Uppsala) according to the manufacturer's instructions. After equilibration in 0.05 M sodium citrate buffer, pH 6.2, 1.5 ml rabbit anti-Dp42 and 3 ml rabbit anti-Dm6 were applied to the columns. Elution of antibodies from the anti-Dp42/Dm-AML column took place with 3 ml equilibration buffer, 3 ml of the same buffer containing 0.5 M sodium chloride and 3 ml 0.05 M sodium citrate buffer, pH 2.6, containing 0.5 M sodium chloride. Fractions of 0.5 ml were collected and monitored in FRIE using swine anti-rabbit IgG antibodies. Fractions 1-6 (absorbed antibodies, denoted anti-Dp42,sp) and 15-18 (antibodies reacting with common epitopes on Dp42 and Dm6, denoted anti-Dp42,com) were pooled.

Antibodies from the anti-Dm6/Dp-AML column were eluted with 6 ml equilibration buffer and fractions 1-6 (each 1 ml) were pooled and denoted anti-Dm6,sp. The second elution step was omitted.

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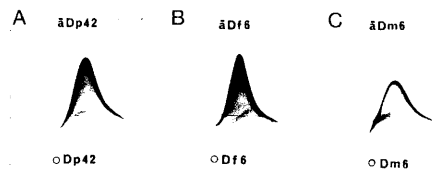


Fig. 1. CIE of purified allergens. A Dp42 of *D. pteronyssinus* (2 µg), rabbit anti-Dp42 (2.86 µl/cm); B Df6 of *D. farinae* (2 µg), rabbit anti-Df6 (2.86 µl/cm); C Dm6 of *D. microceras* (2 µg), rabbit anti-Dm6 (0.3 µl/cm). Agarose: Litex, type HSA. Electrophoretic conditions: First dimension: 10 V/cm for 30 min; second dimension: 2 V/cm for 16 h. Tris-Veronal buffer, pH 8.7, I = 0.02. Plate pressed, washed, dried and finally stained in Coomassie brilliant blue.

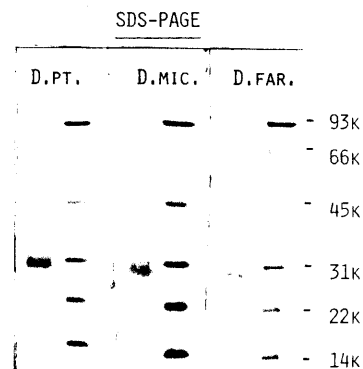


Fig. 2. SDS-PAGE of partially purified antigens Dp42, Df6 and Dm6. Applied: 125 µg AML of each species (left) and MW markers (right). Concentrations of acrylamide and cross-linker (DATD and bisacrylamide) in stacking and separation gel, respectively were: stacking gel: 7T, 5C; separation gradient: 15T, 0.75C - 20T, 1.0C. The MW markers used were: phosphorylase B (93,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soy bean trypsin inhibitor (22,000) and lysozyme (14,000).

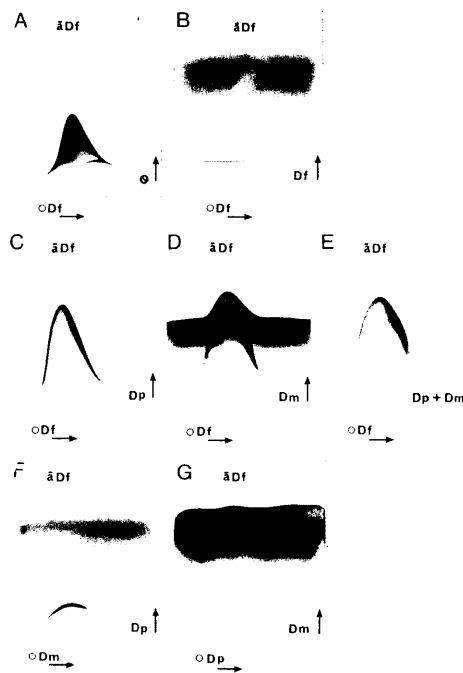


Fig. 3. CLIE experiments, demonstrating partial identity between antigens Dp42, Df6 and Dm6. Antibody: A-E Monospecific rabbit anti-Df6, 1.14 µl/cm; F-G Anti-Df6, 2.29 µl/cm. Antigens applied in first dimension well: A-E 20 µg Df-AML; F 20 µg Dm-AML; G 10 µg Dp-AML. Antigens applied in intermediate gel: A None; B 500 µg Df-AML; C 500 µg Dp-AML; D 500 µg Dm-AML; E 500 µg Dp-AML + 500 µg Dm-AML; F 500 µg Dp-AML; G 2,500 µg Dm-AML. Arrows indicate direction to anode.

Table II. MW and pI

	Dp42	Df6	Dm6
MW, Sephadex G-75	16,500-17,500	16,500-17,500	18,000-19,000
MW, SDS-PAGE	30,000	28,000	29,000
Isoelectric pI's, sucrose gradient	4.5 (5.7)	4.9 (5.5)	4.9 (6.1)
Isoelectric focusing	6.3	6.9	6.5

Results

Purified Df6 and Dm6 appeared as potent immunogens in rabbits. Doses as low as 1.5 µg/kg rabbit stimulated production of high titer monospecific antibodies. CIE of the purified components and their respective antibodies are shown in figure 1. Few weak precipitates demonstrate the presence of small amounts of contaminants.

Determinations of MW and isoelectric points (pI) took place using the partially purified antigen preparations (AML). The elution patterns for the antigens after column isoelectric focusing and gel filtration were easily determined using poly- and monospecific antibodies. In SDS-PAGE one dominating protein band was observed for all three species (fig. 2) corresponding to the position (approximately 30,000) of the previously produced pure antigen Dp42 of *D. pteronyssinus* [7].

Physicochemical characteristics of the three antigens are summarized in table II. The MW and pI are largely identical for the three species and similar to data previously obtained for Dp42 [7]. The allergens of all three species display the same discrepancy between MW as determined by gel filtration and SDS-PAGE and the same degree of pI heterogeneity - two major variants with a distance of approximately 2 pH

units and a nonreproducibly appearing small intermediate peak.

The degree of immunochemical identity between the three corresponding antigens was investigated by CLIE. Examples are shown in figure 3. Summary of the CLIE results appears from table III.

Presence of antibodies to both common and specific determinants was demonstrated by the presence of enlarged precipitates in all 9 experiments with homologous antigen in the well and heterologous antigens in the intermediate gel, either alone or in combination (fig. 2 C-E, table III, rows 3-5). A distinct precipitate was observed when Dm-AML was electrophorized into the anti-Df6 antibody with Dp-AML placed in the intermediate gel (fig. 3F), demonstrating antibody specificity against epitope(s) common to Dm6 and Df6, not shared by Dp42. One further, significantly weaker reaction was observed in the analogous experiments: Dm-6 reacted with anti-Dp42 antibodies with Df-AML placed in the intermediate gel (table III, bottom left).

No precipitate corresponding to the antigen applied in the well was observed in the control experiments with homologous antigen (i.e. of the same species as used to immunize the rabbit) applied in the intermediate gel (fig. 3B). Other experiments with homology between intermediate gel antigen and anti-

Table III. Presence of precipitate in CLIE experiments (in situ antibody absorption)

Antibody ¹								
anti-Dp42			anti-Df6			anti-Dm6		
well ag. ²	line ag. ³	prc.	well ag.	line ag.	prc.	well ag.	line ag.	prc.
Dp	0	+	Df	0	+	Dm	0	+
	Dp	-		Df	-		Dm	-
	Df	+		Dp	+		Dp	+
	Dm	+		Dm	+		Df	+
	Df + Dm	+		Dp + Dm	+		Dp + Df	+
Df	0	+	Dp	0	+	Dp	0	+
	Dm	-		Dm	-		Df	-
Dm	0	+	Dm	0	+	Df	0	+
	Df	+		Dp	+		Dp	-

¹ Monospecific rabbit antibodies to Dp42, Df6 and Dm6; anti-Dp42: 2.9-5.8 µl/cm; anti-Df6: 1.1-2.3 µl/cm; anti-Dm6: 2.3-4.6 µl/cm.

² AML preparations applied in first dimension well; Dp and Df: 10-20 µg; Dm: 100 µg.

³ AML preparations applied in intermediate gel; Dp and Df: 500-1,000 µg; Dm: 1,000-2,500 µg.

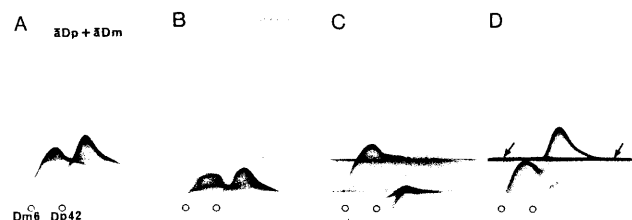


Fig. 4. Tandem CIE with intermediate gel containing antibodies after immunoabsorption. Antigen applied in first dimension wells: A-D 1.0 μ g purified Dm6 (left well), 0.9 μ g purified Dp42 (right well). Antibody in anodical gel: A-D Rabbit anti-Dp42 (0.48 μ l/cm) + rabbit anti-Dm6 (1.2 μ l/cm). Intermediate gel antibodies: A None; B acid eluate (anti-Dp42, com., 10 μ l/cm) from anti-Dp42/Dm-AML column; C neutral eluate (anti-Dp42, sp., 10 μ l/cm) from anti-Dp42/Dm-AML column; D neutral eluate (anti-Dm6, sp., 10 μ l/cm) from anti-Dm6/Dp-AML column. The line precipitate in D (arrows) probably represents Dp-antigen liberated from the Sepharose column during elution.

body or well antigen were considered trivial and were therefore omitted.

The rabbit antibodies, anti-Dp42, sp. and anti-Dm6, sp., obtained after column immunoabsorption, were shown to react with species specificity in CIE when placed in the intermediate gel (fig. 4C, D). The antibodies, obtained by acid elution from the anti-Dp42/Dm-AML column retracted the antigens of both species into the intermediate gel (fig. 4B).

Discussion

Two house dust mite species, *D. pteronyssinus* and *D. farinae*, are reported as frequent inhabitants of house dust in all parts of the world [2]. The occurrence of a third species of the same genus, *D. microceras* [5], is less well investigated, possibly due to the close morphological similarity with the sibling species, *D. farinae*. The discoverers of the species report specimens collected in the UK, Spain and the USA [5].

In the present study, excrement antigens of the two species were purified using procedures similar to the previously reported ones for *D. pteronyssinus* major allergen Dp42. The crucial step is hydrophobic interaction chromatography where advantage is taken of the relatively strong attachment of these molecules to the hydrophobic matrix. The purification effects obtained (with reference to whole culture extracts) were of roughly the same magnitude as the figure (15.6) obtained for Dp42, indicating a 3–10% major allergen weight content in dialyzed culture extracts of the three species.

The almost identical MW and pI data obtained for

Dp42, Df6 and Dm6 (table II) indicate a common molecular structure and origin of the antigens of the three species. In this context, the significant titers of species-specific antibodies demonstrated by CLIE and column absorption experiments are interesting as the species are placed as members of the same genus and two of them (*D. farinae* and *D. microceras*) are regarded as sibling species.

The presented CLIE experiments dealt with antibodies from single rabbits immunized with purified antigens. The immune response of individual rabbits may vary, e.g. due to genetical factors, and give rise to apparently contradictory results such as the demonstration of a common Df/Dm epitope (not shared by Dp) using anti-Df6 antibodies, but not in the analogous experiment using anti-Dm6 antibodies. Species-specific epitopes were, however, unequivocally detected for all three species in the experiments with mixtures of heterologous antigens in the intermediate gel. We have furthermore detected the presence of species-specific antibodies to all investigated species in tandem CIE experiments (through the appearance of distinct spurs) in pools of polyspecific rabbit antisera (results not shown). Data on the species-specificity of house dust mite allergic patients' IgE antibodies will be published separately [8].

The column absorption experiments (including only the species *D. pteronyssinus* and *D. microceras*) demonstrated the possibility of obtaining immunochemical reagents discriminating between antigens of even closely related mite species. We have successfully employed such species-specific reagents in an enzyme-linked immunosorbent assay to study the prevalence of the three species in various areas [10] as

an alternative to the more tedious microscopical examinations.

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