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Antigens and allergens in *Dermatophagoïdes farinae* mite

II. PURIFICATION OF AG 11, A MAJOR ALLERGEN IN *DERMATOPHAGOÏDES FARINAE*

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Summary. Ammonium sulphate precipitation and DEAE chromatography is an efficient way of purifying Ag 11, the main allergen in *Dermatophagoïdes farinae* mites, which has already been characterized by crossed radioimmunoelectrophoresis. At 60% of saturation in ammonium sulphate, a precipitate is formed which, dissolved and dialysed has been named fraction A 60. It is mainly composed of Ag 11. In the fraction DE obtained by DEAE chromatography of the ammonium sulphate fraction A 60, Ag 11 appears homogeneous on crossed-immunoelectrophoresis. Isoelectrofocusing results indicate an average isoelectric point near neutrality in agreement with the non-absorbtion of Ag 11 on the DEAE cellulose at a weak ionic strength (0·01, at pH 7·2). By sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration Ag 11 has a molecular weight of 28,000.

Ag 11 appears as a single polypeptidic chain with numerous dithio-bonds implying a highly folded and resistant structure. Oligosaccharides could be present as constituting molecules as well as contaminating ones as was assumed for hexosamines. These results are discussed with reference to a similar study performed on the major allergen of *Dermatophagoïdes pteronyssinus*. The allergenic properties of Ag 11 as present in fraction DE were tested by RAST-based methods. Fraction DE is an inhibitor as good as Df 80d and when it is coated on paper discs it can bind specific IgE in sera from the majority of mite sensitive patients. The results suggest that Ag 11 is a major allergen from *D. farinae*.

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INTRODUCTION

Some authors isolated and characterized allergenic fractions from Acarian extracts (Romagnani, Biliotti, Passaleva & Ricci, 1972; Romagnani, Boccassini, Amadori & Ricci, 1976; Nakagawa, Kudo, Okudaira, Miyamoto & Honuchi, 1977) and more recently the 'major allergen' of *Dermatophagoïdes pteronyssinus* was defined (Chapman & Platt-Mills, 1978; Chapman & Platt-Mills, 1980).

We have previously shown (Le Mao, Dandeu, Rabillon, Lux & David, 1981) that, using crossed radioimmunoelectrophoresis, the antigen Ag 11 can be characterized in a partially purified *Dermatophagoïdes farinae* extract, Df 80d, as a major allergen since it binds the major part of the mite specific IgE present in a pool of sera from mite sensitive patients. In the present study, the purification of Ag 11 has been achieved. Ammonium sulphate precipitation has been chosen for the fractionation of the partially purified extract because of previous results obtained by other workers (Holford-Strevens, Wide, Milne & Pepys,

1970). The different fractions obtained were characterized by crossed-immunoelectrophoresis, allowing us to prove the presence or the absence of certain antigens among those numbered in the partially purified extract Df 80d. By ammonium sulphate precipitation, three fractions were obtained, one of which, A 60, precipitated at 60% saturation, mainly contains Ag 11. Using ion exchange chromatography on DEAE cellulose, Ag 11 has been isolated. Its physicochemical properties were studied by isoelectrofocusing, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and amino acid analysis. Its allergenic properties were analysed using the radioallergosorbent test (RAST) and RAST inhibition method.

MATERIAL AND METHODS

Allergens

The preparation of the partially purified *D. farinae* extract, Df 80, from a whole mite culture extract provided by Institut Pasteur (Service des Allergènes) has been performed according to the method previously described in detail for *D. pteronyssinus* (Le Mao, Weyer, Pauli, Lebel & David, 1980). The partially purified extract, Df 80, was dissolved in neutral distilled water and exhaustively dialysed against the same medium until the optical density of the dialysate, at 280 nm, disappeared. The resulting brownish solution has been named Df 80d. It was lyophilized and constituted the starting material for further purification.

Antibodies

Immunization procedure was as follows: three rabbits of 2·5 kg received 100 mg of the partially purified extract, Df 80d, in 9 ml of physiological saline solution, mixed with the same volume of Freund's incomplete adjuvant. The resulting emulsion was inoculated subcutaneously in ten points along the spine for one part and a 5 ml inoculation of the same emulsion was done by intraperitoneal route. After 1 month rabbits were boosted with 10 mg of Df 80d in 1 ml of physiological saline solution, by intravenous inoculation. This was repeated 1 month later. One week after the last booster rabbits were bled. Sera were harvested, pooled and concentrated four times.

Ammonium sulphate precipitation

Precipitations were carried out at 4°. Saturated ammonium sulphate solution was progressively added with continuous stirring. Precipitates were allowed to be formed at 4° overnight and then centrifuged. They were washed twice with the same ammonium sulphate concentration used for initial precipitation, dissolved and exhaustively dialysed against neutralized distilled water. The resulting supernatant after the last precipitation was thoroughly dialysed, lyophilized, and dissolved in neutralized water.

Crossed immunoelectrophoretic method

Crossed immunoelectrophoresis (CIE) was essentially performed as described by Axelsen, Krøll & Weeke, 1973. CIE was carried out on 7 cm × 10 cm glass plates: 10–20 μl of each fraction were applied in the well. First and second dimension electrophoresis were performed under 20 V per cm for 30 min and 2 V per cm for 18 hr, respectively. The temperature of the cooling water was 15°. Specified amounts of rabbit antiserum were included in the second dimension electrophoresis gel. Agarose gels were prepared as 1% solution in Tris-Veronal buffer, pH 8·7 (73 mM Tris, 24·5 mM Veronal, 0·36 mM calcium lactate and 0·2 mM sodium azide). After electrophoresis the gels were pressed, washed and pressed again, dried in hot air and then stained in a solution consisting of Coomassie brilliant blue 'R 250', 5 g/litre in ethanol:acetic acid:water (9:2:9). The stained gels were destained in ethanol:acetic acid:water (5:2:9) and dried again.

Ion exchange chromatography

Ion exchange chromatography was performed on DEAE cellulose (Whatman DE 52) equilibrated in 0·01 M NaCl, 0·01 M phosphate buffer at pH 7·2 ± 0·02 and poured in a column 1·6 cm × 40 cm. Elution was carried out in three steps. First, the column was thoroughly rinsed with the buffer used for equilibration, then with a 0·1 M NaCl buffered solution and followed by a linear gradient of ionic strength between 0·1 and 0·5.

Isoelectric focusing

Analytical isoelectric focusing was performed using a LKB Multiphor apparatus and commercially available flat bed plates of polyacrylamide gel (LKB PAG plates of pH range 3·5 to 9·5), according to the manufacturer's instructions. Samples of 10 μl were applied to the gel surface. The PAG plate was focussed at 22 W for 1·5 hr. The gel was then stained for protein identification (Coomassie brilliant blue).

Sodium dodecyl sulphate polyacrylamide slab gel electrophoresis

Allergen preparation (1–5 mg protein/ml) was analysed by this technique as described by Laemmli (1970) using 0·025 M Tris, 0·2 M Glycine buffer pH 8·4 containing 0·1% SDS. The sample (20–50 μl) with 2·5% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0·001% bromothymol blue (tracking dye) in Tris-glycine buffer were boiled for 90 sec in a water bath before being loaded into the gel. Electrophoresis was carried out on a 7·5%–20% gradient slab polyacrylamide gel (10 × 14 cm, 2 cm thick) at 7 mA for 24 hr until the dye reached the bottom of the gel. Sample wells were made from 3% polyacrylamide containing 0·1% SDS. After electrophoresis, the gel was stained with Coomassie brilliant blue.

Estimation of protein concentration

Protein content was quantified on fractions as described by Lowry, Rosenbrough, Farr & Randall (1951) using Folin-Ciocalteau reagent.

Estimation of reducing sugars concentration

Total hexose determination was done on fractions using the Orcinol method.

Estimation of total hexosamines

Hexosamines were determined according to the modified Elson Morgan method (Elson & Morgan, 1933; Antonopoulos, 1962).

Estimation of molecular weights

This was performed by comparing at 280 nm, migration patterns of the fractions studied with those of known molecular markers (blue dextran 2,000,000, ovalbumin 44,000, α-chymotrypsin 23,500, lysozyme 15,000, and phenol red 600) applied to the same Sephadex G 100 column (1·6 cm × 100 cm, M NaCl, 10 mM phosphate buffer pH 7·2 ± 0·02, flow rate 18 ml/hr).

Radioimmunoassays

Radioallergosorbent (RAST) test. The *D. farinae* extract Df 80d or DE fraction were coupled to CNBr-activated paper discs as described by Ceska, Eriksson & Varga (1972). The RAST was performed according to the principle described by Wide, Bennich & Johansson (1967). Serum (50 μl) was incubated with an allergen-coated paper disc 3 hr at room temperature. Then the disc was washed and 50 μl ^{125}I-labelled anti-IgE (Pharmacia, France) were added. After an overnight incubation the disc was washed and counted in a gamma counter for bound radioactivity. The results were expressed as percentage of bound radioactivity.

RAST inhibition. The experiments were carried out essentially as previously described by Le Mao *et al.* (1980). The mite serum pool consisted of equal volumes of serum from sixteen non-hyposensitized subjects who were highly allergic to *D. farinae* mites. The paper discs used were coated with the Df 80d extract, constituting reference discs. The assays were performed as follows: 100 μl of serial dilutions of the extract or of DE fraction were preincubated 3 hr at room temperature with 100 μl of ten-fold diluted *D. farinae* serum pool. Then, these mixtures were used for RAST on reference discs. The point 0% of inhibition was obtained by incubating the serum pool with 100 μl of phosphate-buffered saline. Each inhibition point was performed in duplicate. A dose–response curve was defined by plotting the percentage RAST inhibition versus the varying quantities of the inhibiting extracts.

RESULTS

Ammonium sulphate fractionation of Df 80d

In a preliminary experiment we found that the first precipitation occured when ammonium sulphate concentration reached 50% of saturation. A second precipitation appeared at 66% and a third one at 75%, each precipitate so obtained was dissolved and treated as described in Materials and Methods and they were named respectively A_{50}, A_{66} and A_{75}. We have verified by CIE that Ag 11 is present in the A_{50} fraction as well as in the A_{66} fraction, the latter containing antigens also present in A_{75}. Thus, we have chosen to perform the first precipitation in 60% of ammonium sulphate saturation, assuming that other antigens could be left in the supernatant and totally precipitated in 75% of ammonium sulphate saturation. We carried out fractionation of the partially purified extract Df 80d according to this scheme.

Study of the ammonium sulphate fractions by CIE

For sake of clarity the crossed immunoelectrophoretic reference pattern of Df 80d extract described in a preceding paper is shown again in Fig. 1a. Eleven antigens could be numbered and no cathodal proteins bound to immunoprecipitates were observed. The

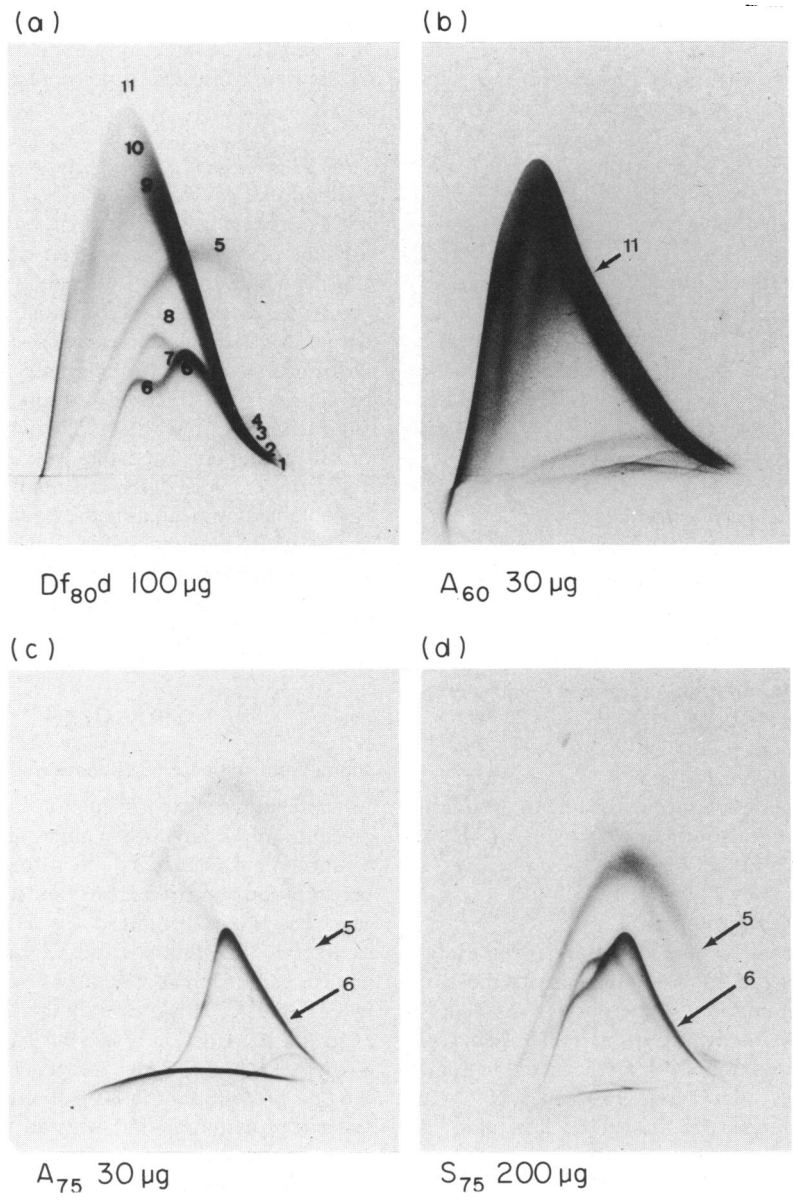

Figure 1. Crossed immunoelectrophoretic patterns of: (a) partially purified *D. farinae* extract; (b) A_{60} fraction; (c) A_{75} fraction; (d) S_{75} fraction. Gel dimensions: 2×7 cm^2, 8×7 cm^2 for the first dimension and the anodic antibody containing gels, respectively.

crossed immunoelectrophoretic precipitation pattern for the fractions A_{60}, A_{75} and S_{75} are shown in Fig. 1. As we can see comparing fractions with Df 80d, A_{60} is enriched in Ag 11 (Fig. 1b) while A_{75} is almost devoid of Ag 11 (Fig. 1c) but is enriched in other antigens, particularly Ag 6. Furthermore, to reveal all the antigenic components (e.g. Ag 5 and Ag 6) of the supernatant fraction S_{75}, it was necessary to use a high concentration of proteins (Fig. 1d).

Purification of Ag 11 by ion exchange chromatography

Since we found by CIE that Ag 11 is slightly negatively

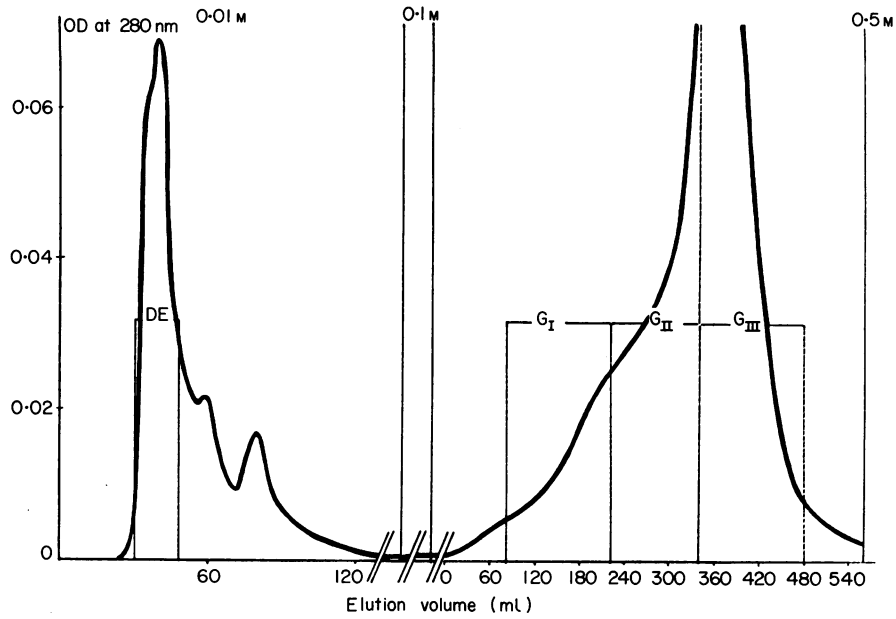

Figure 2. Elution pattern obtained after ion exchange chromatography on DEAE cellulose (column 1·6 × 40 cm; first elution with 0·01 M NaCl, Tris buffer 0·01 M, pH 7·2 ± 0·02; second elution with 0·1 M NaCl, Tris buffer 0·01 M, pH 7·2 ± 0·02; third elution with a linear gradient of molarity from 0·1 M to 0·5 M NaCl, Tris buffer 0·01 M, pH 7·2 ± 0·02, flow rate 18 ml/hr, 3 ml fractions).

charged and other antigens more negatively charged, we chose DEAE cellulose as anionic exchanger. As described in Materials and Methods, we performed a fractionation of A_{60} on DEAE cellulose column. In Fig. 2, it can be seen that four fractions were obtained: DE, G_I, G_{II}, G_{III}. DE is not adsorbed on DEAE cellulose at low ionic strength at pH 7·2, so it would be expected to contain Ag 11 according to its weak negative charge as stated above. The study of the DE fraction by CIE (Fig. 3) showed that it is homogeneous and mainly contains Ag 11. The fractions, G_I, G_{II} and G_{III} contain only traces of other antigens (results not shown).

Physicochemical study of the DE fraction

Since the DE fraction could be considered, from the results described above, as the purest form of Ag 11 obtained up to now, we examined its physical and chemical characteristics. Its approximate molecular weight determined by gel filtration on Sephadex G100, around 27,000 was shown on SDS-PAGE as being 28,000. Ag 11 contains only one peptide chain since it gives only one band on SDS (Fig. 4). It also appeared relatively homogeneous on isoelectric focusing where, as can be seen on Fig. 5, it displays two intense bands with some minor ones. Its homogeneity is obvious in CIE (Fig. 3). The chemical composition of Ag 11 has been studied. Ag 11 contains, using the Orcinol method, 5% of reducing sugars, and 3·7% of hexosamines. The reality of the glycoprotein nature of Ag 11 will be discussed below. The protein moiety of Ag 11 has been analysed by determination of its amino acid

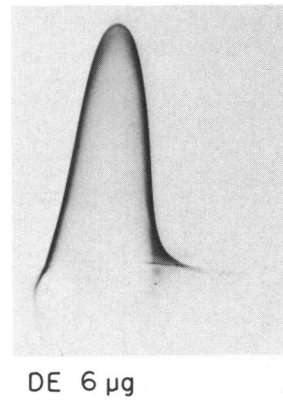

Figure 3. Crossed immunoelectrophoretic pattern of DE fraction. Gel dimensions: 2 × 7 cm^2, 8 × 7 cm^2 for the first dimension and the anodic antibody containing gels, respectively.

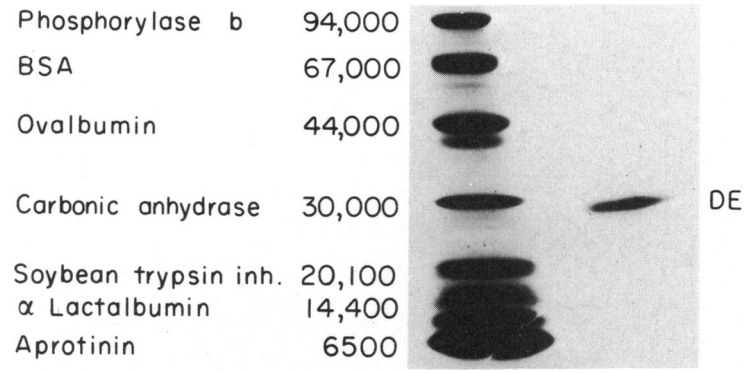

Figure 4. Comparison of the SDS-PAGE patterns of Ag11 and molecular weight markers

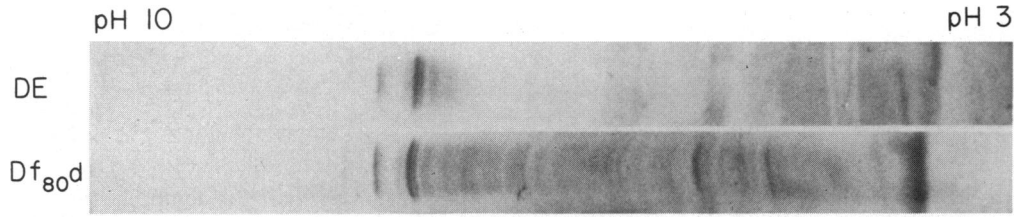

Figure 5. Comparison of the isoelectric focusing patterns of DE fraction and the partially purified *D. farinae* extract.

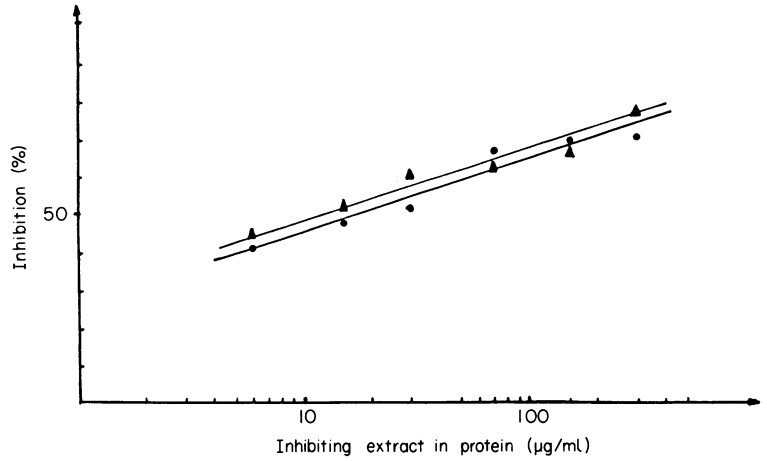

Figure 6. RAST inhibition curves of the partially purified *D. farinae* extract, Df 80d (●———●) and DE fraction (▲———▲) with solid phase of Df 80d.

Table 1. Amino acid composition of Ag 11 in the DE fraction isolated from a partially purified *D. farinae* extract

Residues/100 residues of amino acids	
Asp	:14·20
Glu	:10·91
Gly	:10·98
Ala	: 6·72
Val	: 6·13
Leu	: 4·22
Ileu	: 5·92
Ser	: 6·96
Thr	: 4·46
Cys 1/2	: 4·98
Met	: 1·28
Pro	: 6·75
Phe	: 2·21
Tyr	: 3·32
His	: 3·46
Lys	: 2·91
Arg	: 4·50

Sealed blister, HCl 6 N, 110° under vacuum, 48 hr (protein concentration: 0·6 mg/ml) as determined by the Lowry method.

Table 2. IgE antibody levels* to *D. farinae* extract and to DE fraction by RAST from fifteen patient's sera sensitive to mite.

	Material coated on paper discs	
Serum	Df 80d	DE
1	38	36·6
2	23·6	15·4
3	17·2	9·2
4	9	5·5
5	28·4	12·3
6	29·7	2·9
7	23·6	13·6
8	9·8	3·9
9	21·2	11·8
10	16	7·3
11	30	0·6
12	35·8	2·2
13	20·4	0·8
14	36·2	25·2
15	33·8	20·6
Control†	0·8	0·6

* Expressed as a percentage of bound radioactivity.

† Control: serum pool from non-allergic individuals.

composition. From the results in Table 1, it can be seen that cystine residues are very numerous as well as glycine and alanine residues. The implications of such a chemical composition are considered in the Discussion.

Allergenic properties of DE fraction

Using the RAST inhibition assay, we tested the capacity of DE fraction and Df 80d extract to inhibit the binding of mite-specific IgE from a pool of patient's sera to Df 80d coated paper discs. Figure 6 shows the RAST inhibition results. The slope of inhibition curve produced by DE fraction does not differ from that produced by Df 80d, suggesting a qualitative allergenic similarity between DE fraction and Df 80d extract.

Using the RAST, we compared the level of serum IgE antibodies to Df 80d and to DE fraction in fifteen mite sensitive patient's sera. The majority of them contained IgE antibodies against Df 80d and DE fraction coated on paper discs, as shown in Table 2.

DISCUSSION

The results reported here describe the isolation of Ag 11 previously identified and characterized as an important allergen present in a partially purified *D. farinae* extract (Le Mao *et al.*, 1981). We state here that it can be found as the major part of a protein-rich fraction, precipitated at 60% saturation in ammonium sulphate. This fraction, named A_{60}, has some physicochemical characteristics similar to those described by Chapman *et al.* (1980) for the F_4P_1 fraction isolated from a *D. pteronyssinus* extract, as for example its molecular weight, 27,000, and its very weakly negative charge. The A_{60} fraction has been successfully separated on DEAE cellulose, in four fractions. The first fraction named DE is not adsorbed on cellulose at low ionic strength and neutral pH, and contains a rather homogeneous form of Ag 11. This homogeneity was shown by CIE methods and SDS-PAGE. When reacting with a hyperimmunized rabbit serum against Df 80d, it gives rise to only one precipitin line. Moreover, it must be noted that, if Ag 11 appears as homogeneous in the DE fraction by immunological analysis (Fig. 3), it behaves in isoelectrofocusing as several protein molecules differing by less than one pI unit and gathered around one intense band. Such variation of pI could be well explained by the eventual

formation of amide groups, distribution of which could give rise to weak variations of charge in the same protein molecule (Williamson & Salomon, 1973). Ag 11 can be considered as a single amino acid chain (mol. wt of 28,000) since its molecular weight remains unchanged when it is submitted to dissociating agents in SDS-PAGE. On the other hand, the presence of numerous cystine residues implies the existence of numerous dithio-bonds playing an important role in the cross-linking of different parts in a single peptide chain. Such a particularly many-folded structure could explain its high degree of resistance to denaturation which is obvious, considering the methods used for its preparation i.e. maceration and acetone as well as ammonium sulphate precipitations. When analysed for its amino acid composition, Ag 11 appears as a peptide rich in diacidic amino acid residues which are generally involved in N-acylglycosylamine linkages (Marshall & Neuberger, 1972) and rich in serine and threonine, involved in O-glycosidic linkages (Gottschalk, 1972). Another possibility exists of an O-glycosidic linkage to hydroxylysine (Spiro, 1972). Finally, reducing sugars, the presence of which in the DE fraction has been suggested by the Orcinol method, could also become bound to the protein moiety by the Maillard reaction (Gottschalk, 1972) by formation of N-substituted glycosylamines during the preparative maceration.

So Ag 11 could be a glycoprotein, native or not, and its apparent heterogeneity in IEF could be explained by stepwise removal of sugar residues. Besides, we cannot rule out the possibility of non-covalent interactions between small molecules and proteins which may give rise to apparently different protein species. Such small molecules could have their origin in chitin or in other mite polysaccharides hydrolysed during the preparation of the extract. A protein molecule such as the allergen Ag 11 from *D. farinae*, could be described as 'biosynthetically homogeneous' possibly like immunoglobulins (see Williamson, 1971), and could undergo modifications, due to any of the causes noted above, leading to a 'postsynthetic heterogeneity'. In fact, DE fraction could be heretogeneous and include several different molecules bearing common epitopes, some of which are recognized by human IgE and others by rabbit IgG or both.

Ag 11 as contained in the DE fraction was tested for its allergenic properties by RAST based methods using human specific IgE. Ag 11 in the DE fraction can be considered as rather similar to Ag 11 present in Df 80d since the RAST inhibition curves produced by DE fraction and Df 80d present a parallelism of their slopes. In RAST, a majority of the human patient's sera tested reacts with DE-coated paper discs (optimal DE concentration for fixation on the paper discs was 100 μg/ml). In some cases (patients 6, 11, 12 and 13), antibody level is equal to the control sera or lower than this obtained with Df 80d coated paper discs (optimal Df 80d concentration was 500 μg/ml). A different allergen concentration of the DE and Df 80d fractions on the corresponding discs would lead to a marked difference with a constant ratio between the Df 80d and the DE-RAST values for all the patients. The fact that this result is not observed, points to another explanation for the dissociation of the RAST values: for the majority of the patients studied, the Df 80d-RAST value is one to two and one half times higher than the corresponding DE-RAST result. The observed differences between the RAST values can easily be explained in the following way: for a given serum, the Df 80d-RAST value is the result of the binding of all the available IgE molecules with all the corresponding allergens of the Df 80d fraction, whereas the DE-RAST value is reflecting only the binding between the Ag 11-specific IgE and the corresponding Ag 11 on the disc. The Ag 11- specific IgE molecules constitute only one part of the 'total allergen-components-specific IgE' of these sera and therefore the DE-RAST values can be expected to be lower than the corresponding Df 80d-RAST values. In the rare cases (patients 6, 11, 12 and 13) we can assume that these patients developed little or no IgE antibodies against Ag 11, but certainly possess specific IgE against another allergen present in Df 80d, i.e. the allergen 5 and/or 6 as shown in our previous paper (Le Mao *et al.*, 1981).

To conclude, Ag 11 can be considered as a major allergen of *D. farinae* mite since a majority of patient's sera react with it.

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