

Isolation and Partial Characterisation of Three Major Allergens in an Extract from Cow Hair and Dander

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Abstract. Three 'major allergens' of an extract from cow hair and dander were isolated by means of anion-exchange chromatography and hydrophobic chromatography. Two of the isolated 'major allergens' were immunochemically pure and the third contained less than 1% impurities. The molecular weights were 2.0, 2.2 and 2.4×10^4 daltons, and pI was about 4.0. The amino acid content revealed no specific traits. The protein part of the major allergens constituted about 60% (w/w). The allergenic activity of the isolated allergens was verified in RAST-inhibition experiments and in prick tests.

Introduction

In order to ensure safety and precision in diagnosis and therapy of allergic diseases, the quality and activity of the allergen extracts must be known.

At present, one of the most important tasks in allergy research is to produce extracts containing the 'major allergens' contaminated with as few as possible 'irrelevant' proteins. Using quantitative immunoelectrophoresis we have previously shown that an extract from cow hair and dander contained 17 antigens, of which 4 (Ag 1, 3, 6 and 14) were shown to act as 'major allergens' in humans [31].

In contrast to extracts from dog dander [6], cat dander [28-30] and horse [20-23] dander, only little is known about the composition and potency of that from cow. The present study was undertaken in order to isolate and characterise three of the 'major allergens' in an extract from cow hair and dander in an immunochemically pure and biologically active state.

Materials and Methods

Antigen

An aqueous extract from hair and dander of black and white cattle was made as described previously by Prahl et al. [31]. The extract was made by gently shaking a 100 g/l solution of hair and dander in 0.1 mol/l sodium bicarbonate and 15 mmol/l sodium azide for 24 h at 4°C. After filtration, centrifugation (5.0×10^4 g for

60 min at 0°C) and dialysis (against a 50-fold volume of water for 72 h at 5°C), the extract was freeze-dried and stored dry at 4°C until used as starting material in the fractionations. A 10 g/l solution of this extract in 0.1 M sodium bicarbonate and 0.015 M sodium azide (denoted 'St.C-75') was used for immunoelectrophoretic analysis.

Antibodies

Antibodies against an aqueous extract of hair and dander from four different breeds of cow (black and white cattle, red Danish milk breed, Danish Jersey breed and Charolais), prepared essentially as for St.C-75, were raised in 3 rabbits and purified according to Harboe and Ingild [15]. 10 ml/l of Aprotinin (Novo, Mainz, FRG) was added to avoid proteolytic degradation of the antigens in the second-dimension gel in immunoelectrophoretic analysis [5]. The antibodies were stored at 4°C.

Quantitative Immunoelectrophoretic Methods

Crossed immunoelectrophoresis, crossed-line immunoelectrophoresis and fused-rocket immunoelectrophoresis were performed as described by Axelsen et al. [3]. 10 g/l Agarose (batch 0486, type HSA; Litex, Glostrup, Denmark) in Tris barbital buffer (Tris 0.073 M, barbital 0.023 M, calcium lactate 0.006 M, sodium azide 0.003 M, pH 8.6 at 20°C, ionic strength 0.025) was used for the electrophoresis. The thickness of the first-dimension gel, the intermediate gel and the antibody-containing gel of the electrophoresis was 0.15, 0.12, and 0.10 cm, respectively.

First-dimension electrophoresis was performed for 30 min at 10 V cm⁻¹, and second-dimension electrophoresis for 18 h at 2 V cm⁻¹. Both were performed at 15°C.

Chromatography

The chromatographic separations were performed at 20-22°C, and the pH values of the buffers employed were measured at this temperature.

A peristaltic pump ensured constant flow through the column, and the transmittance of the eluate collected by a fraction collector was monitored continuously at 280 nm. The elution gradients were provided by a gradient mixer (Ultragrad®; LKB, Sweden). Fused-rocket immunoelectrophoresis, which showed the overall immunochemical elution profile and crossed-line immunoelectrophoresis with selected fractions in the line-gel, helped to identify the precipitates corresponding to eluted fractions. The reproducibility of the fractionation procedures was verified by performing all the experiments at least twice.

To ensure an optimal separation with a minimum loss of active material, combinations of the following chromatographic techniques were tried (ion-exchange, hydrophobic, Con A and gel filtration chromatography).

Ion-exchange chromatography: Four ion-exchange resins [CM-, SP-, DEAE- and QAE-Sepharose® (Pharmacia, Sweden) were tested at different pH and ionic strength values according to Lowenstein et al. [22]. The conditions for the preparative chromatography were based on these results. We used one column for each type of resin and for each pH value.

Based upon these experiments, anion-exchange chromatography was carried out on DEAE-Sepharose CL-4B® (Pharmacia, Sweden) with sodium phosphate buffer. The bed volume of the packed column (2.6 × 40 cm) was 210 ml after equilibration with starting buffer. Elution of 2 g of freeze-dried extract from cow hair and dander dissolved in 10 ml of starting buffer was performed at 45 ml h⁻¹ in the following steps: (1) pH 7.6, ionic strength 0.35, 540 ml; (2) a pH gradient: 900 ml, pH 7.6, ionic strength 0.35, mixed with 1,270 ml, pH 5.0, ionic strength 0.35; (3) 540 ml, pH 5.0, ionic strength 0.35; (4) 540 ml, pH 5.0, ionic strength 2.0.

Hydrophobic interaction chromatography was performed on Phenyl-Sepharose® CL-6B (Pharmacia, Sweden). A packed column (2.6 × 40 cm) with a bed volume of 210 ml was equilibrated with ammonium sulphate. The samples were dialysed against 0.005 mol/l ammonium bicarbonate, freeze-dried and dissolved in 10 ml of the equilibrating buffer. The elution was performed at a flow rate of 45 ml h⁻¹ in the following steps: (1) 540 ml, 1.19 mol/l ammonium sulphate washed out unbound proteins; (2) a linear gradient obtained by mixing 3000 ml, 1.19 mol/l with 3,000 ml, 0.19 mol/l ammonium sulphate; (3) 540 ml, 0.19 mol/l ammonium sulphate; (4) 540 ml, 500 g/l ethylene glycol in water; (5) 540 ml aqueous sodium deoxycholate: 20 g/l. The fractions containing the individual major allergens of the extract were pooled separately, dialysed against 0.005 mol/l ammonium bicarbonate, freeze-dried and stored dry at 4°C.

Physical and Chemical Characterisation

The molecular weight of the isolated antigens was determined by means of SDS-polyacrylamide gel electrophoresis according to O'Farrell [27], and by gel chromatography (Ultragel Aca 54®; LKB, Sweden), followed by immunochemical identification [31]. Proteins with known molecular weights (10–160 × 10³ daltons) were used for calibration in both techniques.

pI determination: Isoelectric focusing of the allergen extract was performed using an Ampholine® 8181 column (LKB, Bromma, Sweden). The experimental conditions employed were essentially as described by Lowenstein et al. [22] and Svendsen [34] using Ampholine® pH 3.5–10 and a sucrose gradient from 0 to 5.0% (w/v). The experiment was performed at 18°C and the column was eluted in 60 fractions at a flow rate of 30 ml h⁻¹. The pH values of the fractions were measured and their immunochemical elution profile was

then determined by fused-rocket immunoelectrophoresis. The antigens in question were identified by means of crossed-line immunoelectrophoresis relative to the standard extract. The relationship between the pH values of the eluted fractions and their content of specific antigens determined the pI of these antigens. *Electrophoretic migration velocity* of the major allergens relative to human albumin was determined from the positions of the peaks in crossed immunoelectrophoresis.

Reduction of possible S-S bridges was achieved by boiling the isolated antigens in Tris buffer containing 1,4-dimercapto-2,3-butanediol and sodium dodecyl sulphate. This was then followed by analysis by polyacrylamide gel electrophoresis.

Detergent St. C-75 was dissolved in buffer containing 20 ml/l Triton X-100, and the extract was investigated by crossed immunoelectrophoresis with 20 ml/l Triton X-100 incorporated in the agarose gel.

Denaturation was performed by adding a solution containing: 6 mol/l guanidinium hydrochloride, 0.002 M EDTA, 0.5 mol/l Tris-hydrochloride, pH 8.1, 25°C to each of the isolated antigens. After aeration with nitrogen for 5 min the solution containers were sealed and placed for 25 min in a 37°C thermostatted water bath. Controls were prepared in the same way using the same solution but without guanidinium hydrochloride. The antigens were subsequently analysed by crossed immunoelectrophoresis.

The affinity for Con-A of the antigens was evaluated using free Con-A and Con-A coupled to Sepharose® (Pharmacia, Sweden) in crossed immunoaffinoelectrophoresis according to Bog-Hansen [7] and Bog-Hansen and Brogren [8].

The protein content of the extracts was determined from the optical density at 215 and 225 nm according to Waddell and Chapel Hill [36] and by the method described by Lowry et al. [19], using a Unicam SP-1800 spectrophotometer.

The amino acid composition was determined on a Durrum D 500 amino acid analyser. Hydrolysis was carried out in 6 mol/l HCl at 110°C in evacuated and sealed vials for 24, 48 and 72 h, respectively, according to Moore and Stein [25]. Total cystine-cysteine was determined as cysteic acid after hydrolysis at 110°C for 24 h in 6 mol/l containing 2% of dimethyl sulfoxide in evacuated and sealed vials according to Spencer and Wold [33].

Biological Characterisation

Skin tests were performed as prick tests and the response was estimated according to the Scandinavian Allergy Standard [1]. Histamine dihydrochloride (1 mg ml⁻¹) dissolved in 0.15 mol/l sodium chloride aqueous/glycerol (50% v/v) served as positive control and 0.15 mol/l sodium chloride/aqueous glycerol (50% v/v) as negative control. St.C-75, Ag 1, Ag 3 and Ag 6 were dissolved separately in 0.15 mol/l sodium chloride/aqueous glycerol (50% v/v). Crossed immunoelectrophoresis ensured the same concentration of the isolated 'major allergens' in the reference solution of 'St.C-75' as in the solution of the individual antigens.

RAST-inhibition experiments [2, 12, 13, 37] were performed according to Nielsen et al. [26] with a pool of sera from untreated patients with equally positive reactions in the prick test to Ag 1 and Ag 3. St.C-75 coupled to paper discs served as reference. The inhibition experiments were performed with St.C-75 and the isolated 'major allergens' undiluted and diluted by a factor of 10⁵ in steps of one decade. The measurements were performed twice in duplicate. The concentrations of the isolated antigens determined by immunoelectrophoresis were equal to their concentrations in St.C-75.

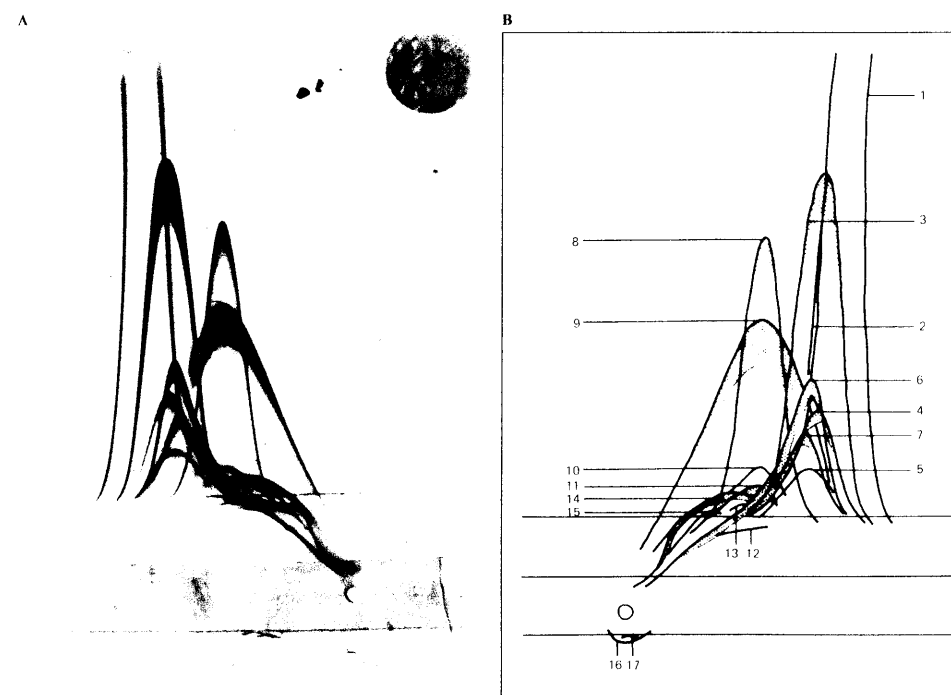


Fig. 1. A Crossed immunoelectrophoretic precipitation pattern for an extract from cow hair and dander ('St.C-75'). Gel: agarose 10 g/l. Buffer: Tris 0.073 mol/l, barbital 0.024 mol/l, calcium lactate 0.006 mol/l, sodium azide 0.003 mol/l (pH 8.6 at 20°C). Samples: 5 µl St.C-75, (10 g/l). Antibodies: rabbit-anti-St.C-75, 8 µl/cm². First-dimension electrophoresis: 0.15 cm thick gel, 10 V/cm for 30 min; anode to the right. Second-dimension electro-

phoresis: 0.13 cm thick intermediate gel, containing no antibodies, has been interposed. Below the first-dimension gel and above the empty intermediate gel: 0.10 cm thick antibody-containing gel, 2 V/cm for 18 h at 15°C, anode uppermost. B Drawing of the same crossed immunoelectrophoretic precipitation pattern. Classification numbers for the precipitates are indicated.

Patients and Sera

10 patients with allergic asthma to cow diagnosed by anamnesis, cutaneous tests, RAST and bronchial provocation tests were used for prick tests. 5 persons with no previous history of allergy and with normal serum IgE served as controls.

Serum samples were obtained from the patients and the controls and were kept frozen (-20°C) until analysed.

Results

Rabbit antibodies were raised against an aqueous extract from cow hair and dander, and a reference precipitation pattern was established by crossed im-

muno-electrophoresis. The extract contained 17 antigens of which 2 moved towards the cathode (Fig. 1). Crossed radio-immunoelectrophoresis has shown 4 of the antigens to be 'major allergens' (antigens No. 1, 3, 6 and 14). Antigen No. 14 has been identified as IgG [31]. As the method for isolating IgG is well known we will not discuss this further.

Isolation of the Major Allergens (Fig. 2)

Ion-exchange chromatography on DEAE-Sepharose CL-4B separated the extract into two main fractions by the use of a pH gradient (pH 7.6 to pH 5.0) followed by a salt gradient (ionic strength 0.35–2.0).

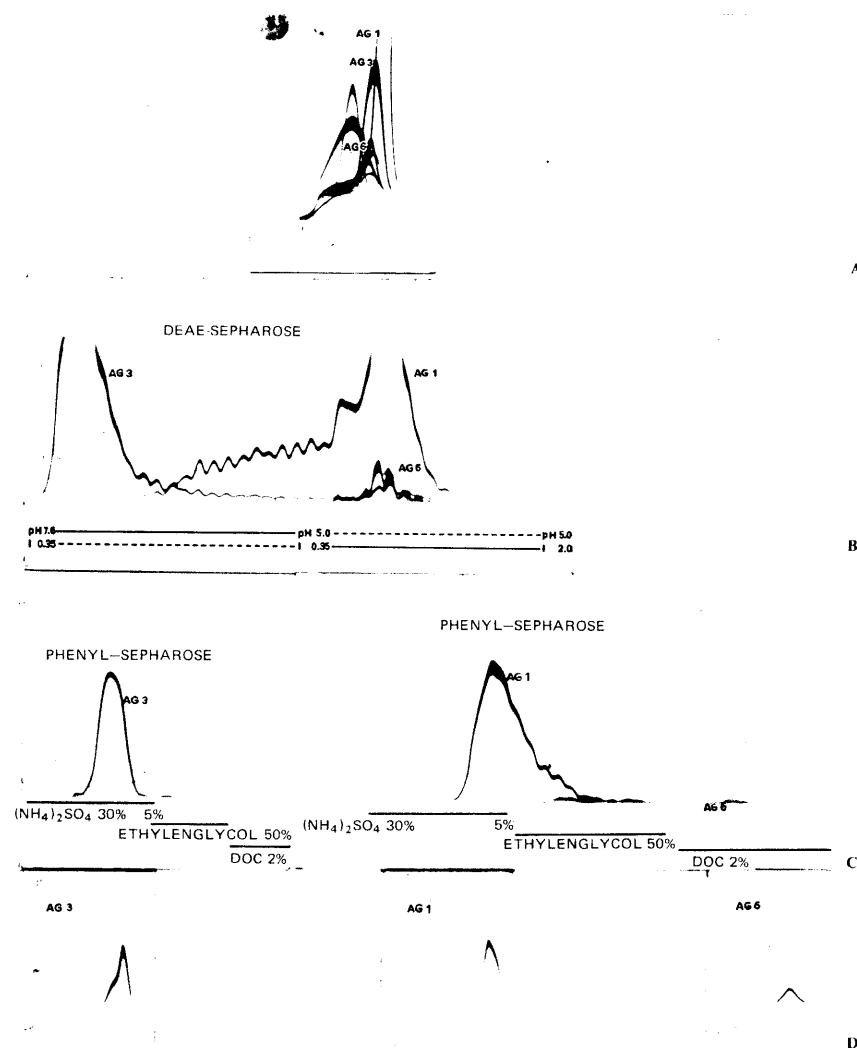


Fig. 2. A The reference precipitation pattern of St.C-75. B Fused-rocket immunoelectrophoresis of fractions of St.C-75 eluted from a DEAE-Sepharose column. 2 μ l of each fraction was applied in adjacent wells in a 0.15 cm thick agarose gel and allowed to diffuse for 1 h before electrophoresis at 2 V/cm into a 0.12 cm thick gel containing rabbit antibodies (3 μ l/cm²) against cow hair and dander. Anode uppermost. C Fused-rocket immunoelectrophoresis of

fractions from chromatography on Phenyl-Sepharose of the two major fractions obtained by an ion-exchange chromatography (B), one containing antigen No. 3, the other antigens No. 1 and 6. The volumes of antigen and antibody used were as in B. D Crossed immunoelectrophoresis of isolated major allergens of St.C-75 antigen No. 1: 1 μ g; antigen No. 3: 1 μ g; and antigen No. 6: 1 μ g against rabbit anti-cow hair and dander (4 μ l/cm²).

Table 1. Physico-chemical characterisation of three isolated major allergens from cow hair and dander

Allergen No.	Protein content % (w/w) ^a		Approximate molecular weight, daltons ^b		Iso-electric point pI ^c	Migration velocity ^d	Recovery % ^e	Antigenic protein impurities, % ^f
	Lowry	OD _{215-225 nm}	AcA-54	PAGE				
1	50	42	2.4×10^4	2.5×10^4	3.6	1.90	20	0
3	55	60	2.0×10^4	2.2×10^4	4.2	1.81	25	0.1
6	-	-	2.2×10^4	2.2×10^4	4.6	1.72	5	0

^a According to Lowry et al. [19] and Varga [35].

^b Determined by Ultrogel AcA-54 gel filtration and by polyacrylamide gel electrophoresis (PAGE).

^c Determined by column isoelectrofocusing using sucrose gradient.

^d Determined in crossed immunoelectrophoresis relatively to human albumin.

^e Estimated by quantitative immunoelectrophoresis.

^f Estimated by quantitative immunoelectrophoresis and by determination of protein content according to Lowry et al. [19].

One fraction contained antigen No. 3, and the second antigens No. 1 and 6. Judged from quantitative immunoelectrophoresis the recovery of the antigens was only about 40%; this low recovery might have been caused by discarding of some fractions because of overlapping and failure to desorb all the material from the separation column. The two pools were dialysed, freeze-dried and dissolved in the starting buffer used for hydrophobic interaction chromatography.

Phenyl-Sepharose was chosen for hydrophobic interaction chromatography. After each of the fractions from DEAE-Sepharose chromatography had passed the Phenyl-Sepharose column, separation was complete as for antigens No. 3 and 6. When the purified antigens were examined by polyacrylamide gel electrophoresis, antigens No. 3 and 6 were found to be almost pure, whereas antigen No. 1 contained a great deal of material with a molecular weight of about 10^5 . After AcA 54 gel chromatography, polyacrylamide gel electrophoresis showed that this impurity was removed. The impurity was not precipitated by rabbit antibodies against cow hair and dander, and it did not cause reactions in prick tests performed on patients with allergy to cow. Judged by quantitative immunoelectrophoresis the recovery of antigens No. 1 and 3 was about 50%, but only 10% for antigen No. 6. The low recovery may be explained as mentioned above.

Fractions containing the major allergens were pooled, dialysed, freeze-dried and dissolved in 1 g/l sodium bicarbonate for further experiments. Using quantitative immunoelectrophoresis, the solution concentration of the isolated antigens were adjusted

Table 2. Amino acid composition of two major allergens from an extract of cow hair and dander

Amino acid	Antigen 1 mol amino acid/ mol protein	Antigen 3 mol amino acid/ mol protein
Asp	28	19
Ihr	14	14
Ser	10	7
Glu	33	31
Pro	7	9
Gly	23	13
Ala	18	8
Cys	4	5
Val	12	9
Met	3	5
Ile	11	12
Leu	14	15
Tyr	10	11
Phe	11	3
His	5	1
Lys	12	9
Arg	7	8

The molecular weights used for the calculations were: antigen No. 1, 2.4×10^4 and antigen No. 3, 2.0×10^4 daltons. Tryptophan was not determined.

to be equal to those in the reference extract. Crossed immunoelectrophoresis revealed antigens No. 1 and 6 to be immunochemically pure, whereas the remaining impurities of antigen No. 3 constituted less than 1%, as judged by quantitative immunoelectrophoresis. Using crossed immunoelectrophoresis, the impurities were identified as antigens No. 5 and 9.

Table III. Results from prick-test¹ and RAST-inhibition² experiments with whole extract and with purified allergens³

Allergens	Prick test (%) in											RAST-inhibition %
	5 normal controls	10 patients										
		PP	NR	GE	HK	GK	ND	AP	BA	KL	GL	
St.C-75	0	100	100	100	100	100	100	100	100	100	100	100
Antigen No. 1	0	60	116	80	13	77	84	51	109	185	131	67
Antigen No. 3	0	77	20	200	83	97	84	100	24	30	125	67
Antigen No. 6	0	0	20	26	13	17	31	30	7	30	56	10
Antigens No. 1+3												83

¹ The activity of the individual allergens is given relative to that of the whole extract:

$D \times d \text{ antigen} \times 100$ %, where D and d are mutually perpendicular diameters of the urticaria element.

² The maximum inhibitory capacity of the extracts is given relative to the maximum inhibition caused by St.C-75. Serum pool: PP, GK, ND, G4.

³ The purified allergens had the same concentration as they had in the whole extracts.

Determination of Physicochemical Characteristics of the Isolated Three Major Allergens of Cow Hair and Dander

The molecular weights were estimated both from gel chromatography and from polyacrylamide gel electrophoresis with SDS. All molecular weights were about 2×10^4 (table I). The results obtained by the two methods were concurrent. The pI values of antigens No. 1, 3, and 6 were determined to be 3.6, 4.2, and 4.6, respectively (table I). All three 'major allergens' migrated faster towards the anode than human albumin.

Amino acid analysis: The amino acid compositions of antigens No. 1 and 3 are shown in table II. Owing to shortage of material, No. 6 was not investigated. Amino acid analysis failed to reveal any unusual amino acid composition relative to common mammalian proteins. On the basis of protein content determined according to Lowry et al. [19] and quantitative immunoelectrophoresis, the relative protein content of the individual antigens in the whole extract was determined. The results are given in table I together with the determination of protein contents. The reference extract and antigen No. 3 contained 60% protein. Antigen No. 1 contained only about 50%.

S-S bridges and non-covalent bonds: In order to see whether the antigens consisted of peptide chains held together by S-S bridges, they were examined by SDS-polyacrylamide electrophoresis before and after reduction using dithiothreitol. The position of the bands remained unchanged.

Exposure to guanidinium hydrochloride (6 M) altered the precipitation pattern of all of the antigens. The precipitates became a little less blurred and retarded in migration.

Detergent added to the extract changed the precipitation pattern of antigen No. 6. Part of the antigen precipitate was not affected while another part appeared in a more cathodic position. Judging from the peak areas, the latter component constitutes the major part of the molecules of this antigen. Further, a new precipitate was seen below the cathodic part of the precipitate for antigen No. 6. This precipitate showed partial immunological identity with the anodic limb of the latter.

Reaction with Con A showed antigens No. 1 and 6 to be heterogeneous molecules with varying affinity for Con A. A detailed description is given by Bøg-Hansen et al. [9].

RAST inhibition. The total inhibition shown by the isolated antigens and by antigens No. 1 and 3 used together was measured. Each antigen only partially inhibited the binding of specific IgE to the antigens of St.C-75. On the other hand, antigens No. 1 and 3 seem to account for most of the allergenic activity of the extract. The results of the experiments are given in table III.

Prick test. When the purified antigens were tested in vivo by the prick tests, the three 'major allergens' showed fully retained allergenic activity. All 10 patients reacted to antigens No. 1 and 3 while 2/10 pa-

tients showed no wheal when they were tested with antigen No. 6. The degree of sensitivity to each of the antigens varied from patient to patient. The results are summarised in table III. In most cases the sum of the allergenic activities exhibited by the individual isolated antigens was greater than 100%.

Discussion

In this study we have described how three 'major allergens' in an extract from cow hair and dander can be isolated. On the basis of systematic studies, ion exchange chromatography on DEAE-Sepharose was chosen as the most useful separation procedure, followed by hydrophobic chromatography on Phenyl-Sepharose. With the latter technique, proteins are separated according to differences in their hydrophobic groups.

Antigens No. 1 and 3 were isolated with a low but acceptable recovery of 20%. However, the very low recovery of antigen No. 6 prevented us from purifying enough material for further experiments.

The molecular weights of the major allergens were estimated by two methods, giving concordant results. The estimated values are also consistent with those calculated on the basis of thin-layer-chromatography experiments and ultracentrifugation [16, 32]. As antigens No. 1 and 6 are glycoproteins it may well be that their molecular weights are overestimated because of the globular shape of glycoproteins.

All three major allergens had acidic pI values, a finding which is in accordance with previous reports, thus indicating the acidic nature of mammalian allergens [4, 10, 18, 22, 30, 35].

The amino acid analysis of cow antigens No. 1 and 3 did not reveal any resemblance to the antigens isolated from horse hair and dander [22]. The amino acid sequences are known for four allergens [11, 14, 17, 24] and the sequence data do not reveal any unusual features.

The protein content was determined for antigens No. 1 and 3 by the Lowry method, and compared to results obtained by measurements of the optical density at 215 and 225 nm. The results correlated well for Ag 3, whereas there was some disparity in the results for Ag 1. Since carbohydrate is known to exert an inhibitory effect on the Lowry protein assay and antigen No. 1 contains carbohydrate, it is possible that the

protein content of allergen No. 1 has been underestimated in this assay.

We found no evidence to indicate that the allergens are peptides held together by S-S bridges. Guanidinium ion produced generalised heterogeneity with regard to migration velocity. The retardation seen is due to neutralisation of negative charges, and the blurred precipitates might indicate that the antigens consist of more than one chain held together by non-covalent bonds, which are neutralised by guanidinium ion. The use of a non-ionic detergent (Triton X-100) in the gel caused a retardation and change of the precipitation pattern of antigen No. 6. The retardation arises as a result of micelle formation, which involves a reduction in the charge density. The new precipitate seen after addition of detergent is probably due to a peptide occluded in the molecule of allergen No. 6 by hydrophobic bonds.

The allergenic activity of the isolated allergens relative to that of St.C-75 was determined by means of RAST-inhibition experiments and prick tests. Both methods revealed that antigens No. 1, 3 and 6 together displayed approximately the same allergenic activity as the whole reference extract (St.C-75). However, antigen No. 1 and 3 together showed nearly as much, thus verifying the results of our earlier 'CRIE studies', which indicated antigens No. 1 and 3 to be the most important allergens of our extract from cow hair and dander [31].

The prick tests showed great individual variations in the patients' sensitivity to the 'major allergens', a finding which is in accordance with previous results obtained with CRIE [31]. In most cases the sum of the activities displayed by the individual allergens was greater than that for extract as a whole. This might be due to an inhibitory effect exerted by the other proteins of the whole extract.

Considering these results as well as the very low recovery from purification of antigen No. 6 it was considered impractical to attempt to isolate antigen No. 6 for further experiments.

The allergens were isolated immunochemically pure and biologically active. In general, the results of in vitro and in vivo diagnosis and of immunotherapy are very often doubtful, and the possibility that the use of purified 'major allergens' can improve diagnostic procedures is currently being investigated. Further, the occurrence and quality of allergen-specific IgG and IgE will be shown. By using purified major allergens it might be possible to immunise with higher

doses of the offending allergens and through this perhaps improve the results of immunotherapy with extract from cow hair and dander.

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