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Characterization and Chemical Modification of Isolated Allergens from Horse Hair and Dandruff

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Abstract. Three previously isolated major allergens of horse hair and dandruff, antigens No. 6, 9 and 11 (Ag 6, 9 and 11) have been further characterized and chemically modified. Carbohydrate analyses revealed that Ag 9 and 11 contained 12 and 17 hexoses, amino sugars, sialic acids as well as low amounts of unidentified sugar molecules. The carbohydrate part of the glycoproteins constituted 5 and 10% (w/w) of dry-weight material. Addition of the non-ionic detergent Berol did not change the molecular structure of the three allergens. Denaturation using 6 M guanidinium hydrochloride was reversible for Ag 6 and 11, whereas Ag 9 separated into two non-identical molecules of almost equal size. Several derivatives of the three allergens coupled to ethylene maleic anhydride copolymer (EMA) were prepared by coupling the amino groups of the allergens (molecular weight EMA: 20,000 daltons). The recovery of the prepared water-soluble complexes was greater than 85% in all experimental cases studied and the complexes obtained were from one to several EMA and allergen molecules; both intra- and intermolecular bridges were established. Immunochemical analyses revealed that through attachment of EMA chains, antigenic sites were masked. The preserved activity of modified relative to native allergens was from 1 to 31% as measured by means of single radial immunodiffusion (on the basis of polyspecific rabbit antibodies against the allergens), RAST inhibition (on the basis of human IgE antibodies from 22 horse-allergic patients), and prick tests (on the basis of the individual reactions of 4-6 horse-allergic patients).

Introduction

The use of chemically modified allergens or allergen extracts has recently been reviewed in terms of allergoids by Marsh [1975] who modified rye grass pollen group I antigen and whole-pollen extracts by introduction of intra- and intermolecular

methylene bridges using formaldehyde. The main product was a mixture of dimers and trimers. Patterson *et al.* [1973a, b]; Patterson and Suszko [1976] modified ragweed AgE by means of the same principle using glutaraldehyde and obtained complexes containing from 5 to 500 AgE molecules. King [1976] also modified AgE either by at-

taching large-molecular-weight groups as dextran or methoxypolyethylene glycol or by simply changing the conformation of the native allergen molecule by physical or chemical methods. At present, studies involving these modified allergens are not yet completed, but preliminary results obtained show that it is possible to treat patients with higher doses of modified allergens than of native allergens and that the modified allergens in most cases have reduced allergenicity.

Using ethylene maleic anhydride copolymer (EMA) as an agent, it is possible to modify protein even when it contains only a few reactive amino groups [Løwenstein, 1974]. By this modification, it is possible to obtain water-soluble polyanionic derivatives over a very wide molecular range held together by the polyanionic flexible chains which may also establish intrachain bridges.

Katchalsky [1970] has shown that the attachment of flexible polyanionic chains to proteins changes the electrostatic micromilieu which is partially responsible for the immunogenicity of proteins. Furthermore; the established intra- and intermolecular chains may give rise to a different conformation on which the immunogenicity also depends.

The availability of well-characterized isolated allergens of horse hair and dandruff [Løwenstein *et al.*, 1976b] allows the modifications to be carried out under controlled conditions and also the study of the physical and immunochemical changes at a molecular level.

Besides a further characterization of these allergens, the purpose of this study is, to modify allergens by means of EMA, to determine the degree of modification as well as the preserved activity of the modified allergens, relative to that of the native allergens.

Materials and Methods

Allergens, antigens No. 6, 9 and 11 (Ag 6, 9 and 11) of horse hair and dandruff were isolated and quantified as described by Løwenstein *et al.* [1976b].

Rabbit antibodies against whole extract of horse hair and dandruff were obtained as described by Markussen *et al.* [1976].

Quantitative immunoelectrophoretic methods including crossed immunoelectrophoresis (CIE), crossed-line immunoelectrophoresis (CLIE), fused rocket immunoelectrophoresis and rocket immunoelectrophoresis were performed essentially as described by Axelsen *et al.* [1973].

CIE of Ag 6, 9 and 11 dissolved in detergent [1% (w/v) Berol, EMU-043; Stenungssund, Sweden], in detergent-containing agarose gels was performed according to Bjerrum and Lundahl [1973].

Carbohydrate analyses were carried out by means of gas-liquid chromatography (GLC) according to Sawardeker *et al.* [1965] on Ag 9 and 11. The carbohydrates of the glycoproteins were hydrolyzed for 18 h in 0.5 N sulfuric acid at 100 °C, neutralized with barium carbonate, reduced to corresponding alditols by means of sodium borohydride using xylose as internal standard, acetylated and transferred to chloroform. The carbohydrates were separated in a Pye 104 gas chromatograph (Cambridge, England) using a 6 ft × 4 mm column packed with Gaschrom Q and coated with ECNSS-M (App. Science Lab., State College, Pa.). Preparations of acetylated alditols and GLC using xylose as internal standard were both performed in duplicate. The range of retention times and quantitative determinations obtained were in all cases within ± 2 and ± 8%, respectively. Amino sugars were semiquantitatively determined by the same procedure after previous formolysis.

Denaturations were performed by adding 0.1, 1.0, and 1.0% (w/v) Ag 6, 9, and 11, respectively, to the following solution: 6 M guanidinium hydrochloride, 0.002 M EDTA, 0.5 M Tris-hydrochloride (pH 8.1, 25 °C). These solutions were aerated with nitrogen for 5 min, sealed and placed for 25 min in a 37 °C thermostated water-bath (A). Controls (B) were prepared in the same way using a solution not containing guanidinium hydrochloride

ride and, together with A, stored at -20 °C before CIE analysis.

Reduction of possible S-S bridges of Ag 6, 9 and 11 was performed by adding about 200 mol 1,4-dimercapto-2,3-butandiol per mole dissolved, denatured protein (A), aerating with nitrogen for 5 min and continuing the reaction in sealed vessels for 2 h at 37 °C. The blocking of free sulphhydryl groups was performed in the dark by adding about 500 mol iodoacetamide per mole dissolved, reduced protein which was aerated with nitrogen for 5 min, sealed and finally placed in a water-bath (37 °C) for a further 25-min reaction before storage at -20 °C. The final products were subjected to CIE analysis.

Covalent coupling of linear EMA [grade 11, molecular weight 20,000 daltons; Monsanto, La Salle, Que., Canada; Svenson, 1974] to solutions of Ag 6 (1 mg), Ag 9 (10 mg) and Ag 11 (10 mg) was performed in 2 ml Tris-hydrochloride (ionic strength 0.2, pH 8.8, 0 °C). The reactions were carried out in a pH-stat (Radiometer; Copenhagen, Denmark) at 0-2 °C, pH 8.8, keeping the pH constant by adding 2 M sodium hydroxide. Varying amounts of EMA were added and the reactions stopped when no further uptake of sodium hydroxide could be detected. Controls without protein or EMA were also carried out. No precipitation occurred during the reaction.

Isolation of EMA-coupled Ag 6, 9 and 11 was performed in the following steps: (1) precipitation using an excess of 0.5 M calcium chloride; (2) 5 times centrifugation (50,000 g, 10 min, 0 °C) and subsequent washing of the precipitate in the reaction buffer containing 0.5 M calcium chloride; (3) dialysis of precipitate overnight against 100 ml 0.1 M EDTA at 4 °C; (4) dialysis overnight against 100 ml 0.1 M sodium chloride, 0.05 M sodium hydrogen carbonate; and (5) storage at -20 °C.

Unmodified Ag 6, 9 and 11 did not precipitate with an excess of 0.5 M calcium chloride and all the precipitated material obtained after step 2 was dissolved when dialyzed against EDTA in step 3. Consequently, the amounts of unmodified Ag 6, 9 and 11 were determined immunochemically during isolation step 2 using rocket immunoelectrophoresis.

Molecular weights of EMA-coupled allergens and controls were determined by gel filtration us-

ing Ultrogel ACA 22 as described recently [Løwenstein *et al.*, 1976b].

Single radial immunodiffusion (SRID) of EMA-coupled allergens and controls against rabbit anti-horse hair and dandruff antibodies (8 µl · cm⁻² gel area) was performed in 1% (w/v) agarose gel containing 0.073 M Tris, 0.024 M barbital, 0.006 M calcium lactate, 0.003 M sodium azide (pH 8.6, 25 °C) and 5% (v/v) glycerol according to Mancini *et al.* [1965]. Volumes of 5 µl of serial dilutions of samples and controls were applied in the wells and incubated for 96 h at 22 °C. The analyses were performed twice at four different antigen concentrations. After staining with Coomassie brilliant blue [Axelsen *et al.*, 1973], the diameters of the precipitin rings were measured with an accuracy of 0.5 mm. The preserved antigenic activity of EMA-coupled allergen relative to native allergen was calculated from the areas enclosed by precipitin rings corresponding to protein amounts applied. The ranges of the results were within ± 10% of the calculated mean values.

RAST inhibitions [Foucard *et al.*, 1972] were performed using serial dilutions (2 to 2 × 10⁻⁴ mg · ml⁻¹ in semilog₁₀ steps) of corresponding protein amounts of native and EMA-coupled allergens for the inhibition of the serum IgE binding to native allergen-coupled (10 mg · ml⁻¹) paper discs. A pool of equal amounts serum of 22 horse-allergic patients was used [Løwenstein *et al.*, 1976a]. The preserved allergenic activity of EMA-coupled allergens relative to native allergens was expressed as the concentrations corresponding to 50% inhibition in percent

$$\frac{C_{\text{native}}^{50\%}}{C_{\text{EMA-modified}}^{50\%}} \times 100$$

[Nielsen *et al.*, 1974]. The determinations were performed twice in duplicate and the ranges of results were within ± 25% of the mean value.

Prick tests were performed on untreated patients allergic to St H 75 from the out-patients' clinic for adults, University Hospital, Copenhagen, as described by Løwenstein *et al.* [1976b]. Histamine dihydrochloride (1 mg · ml⁻¹) and solvent [1:1 (v/v) solution of glycerol and 0.154 M sodium

chloride] containing 1 mg·ml⁻¹ hydrolyzed EMA were used as positive and negative controls, respectively. The preserved allergenic activities of EMA-coupled relative to native allergens in corresponding protein amounts were expressed in percent.

Results and Discussion

Partial characterization including molecular weight, pI, protein, and amino acid analyses of Ag 6, 9 and 11 has recently been reported [Løwenstein *et al.*, 1976b]. The study suggested a significant content of carbohydrate in Ag 9 which we have not been able to verify (table I). Ag 9 contained about 2 desoxyhexose and 10 hexose units and semiquantitatively low amounts of unidentified amino sugars were also detected; together they constituted about 5% (w/w) of the dry-weight glycoprotein. Compared to Ag 9, Ag 11 contained high amounts of hexoses, especially mannose, and together with the semiquantitatively determined amino sugar it constituted at least 10% (w/w) of

the dry-weight glycoprotein, corresponding to about 17 sugar molecules attached to this molecule. This result corresponds well with the carbohydrate analyses performed by Stanworth [1957] on an isolated horse dander allergen (molecular weight 34,000 daltons) which is undoubtedly identical with Ag 11. Whereas Ag 9 and 11 contained only insignificant amounts of unidentified carbohydrates, we may infer from the results of neuraminidase treatment [Markusen *et al.*, 1976] that Ag 11 contains significant amounts of sialic acid. In the above analysis, the influence of the various separation steps used for the isolation of Ag 9 and 11 as well as contamination of carbohydrates originating from the Sephadex and/or Ultrogel columns used cannot be ignored; the former is caused by partial degradation of various carbohydrates of the glycoproteins during ionic exchange and gel filtration, whereas the latter only influences the glucose and galactose determinations since dextrans (Sephadex) and agarose (Ultrogel) are polymerization products of these sugars.

Table I. GLC carbohydrate analysis of Ag 9 and 11

	Retention time relative to xylose			Carbohydrate content relative to dry weight % (w/w)	
	standards	Ag 9	Ag 11	Ag 9	Ag 11
Fucose	0.51	0.51	—	0.7	—
Mannose	1.86	1.87	1.86	1.03	8.1
Galactose	2.17	2.11	2.17	1.63	1.6
Glucose	2.45	2.46	—	0.89	—
Unidentified		0.38; 0.54; 0.61; 0.74	0.35; 0.44; 0.58; 0.65; 1.10; 1.36; 1.51		
Amino sugar (semiquantitative)				0.3	0.5

Detergent added to Ag 6, 9 and 11 did not change their CIE precipitation pattern as compared with the native molecules. Denaturation by means of 6 M guanidinium hydrochloride was found to be completely reversible for Ag 6 and 11 when subjected to CIE analysis, whereas Ag 9 degraded partially to an immunologically partially identical antigen of relatively higher mobility. This was identified as Ag 7 which has recently been shown to emerge during isolation of Ag 9. Ag 7 has a molecular weight of 25,000 daltons, binds IgE from horse-allergic patients, and, contrary to Ag 9, it is not partially identical with dander antigens from other mammalian species [Løwenstein *et al.*, 1976b]. Thus it is likely that Ag 9 is composed of two nonidentical polypeptide chains held together by non-covalent forces; only one of these chains has antigenic sites essentially different from those of the corresponding mammalian molecules. CIE ana-

lysis of molecules after reductive degradation of S-S bridges and subsequent blocking of free SH groups with superfluous reagents performed on native Ag 6, 9 and 11 revealed only insignificant qualitative and quantitative changes. Together with the low content of half-cystine as a result of the amino acid analysis [Løwenstein *et al.*, 1976b], it is thus shown that none of the major allergens of horse hair and dandruff are stabilized by interchain S-S bridges.

The chemical coupling of EMA to native allergens was performed with various proportions between the reactants (except for Ag 6) as shown in table II and the method of isolation used ensured against contamination with the native allergens. The products obtained were all easily dissolved. The recovery, the molecular-weight ranges, and the weight averages of EMA chains attached to the allergens were not influenced by the various proportions between the reactants.

Table II. EMA modification of allergens of horse hair and dandruff

No.	Allergen	Protein/EMA proportions during reactions, mole:mole	Recovery of modified ¹ protein % (w/w)	Approximate molecular weight ranges ² of EMA/allergens, daltons	EMA/allergen ³ mole:mole
1	Ag 9	1:32	93	80,000–180,000	1.7
2	Ag 9	1:8	90	70,000–180,000	1.7
3	Ag 9	1:2	90	80,000–210,000	1.8
4	Ag 6	1:16	85	40,000–160,000	1.5
5	Ag 11	1:64	98	90,000–210,000	1.6
6	Ag 11	1:16	96	50,000–210,000	1.6
7	Ag 11	1:4	94	50,000–160,000	1.5

Molecular weights of Ag 6, 9 and 11 are 19,000, 51,000 and 31,000 daltons, respectively [Løwenstein *et al.*, 1976b].

¹ Determined from the preserved amounts of unmodified allergens by means of rocket immunoelectrophoresis.

² Determined by Ultrogel ACA 22 gel filtration using native allergens and human serum proteins of known molecular weights as references (fig. 1).

³ Estimated from the A 280 nm – absorption curve profile obtained during elution of EMA – modified allergen from the gel filtration matrix neglecting the light absorption of the EMA-carboxylic groups.

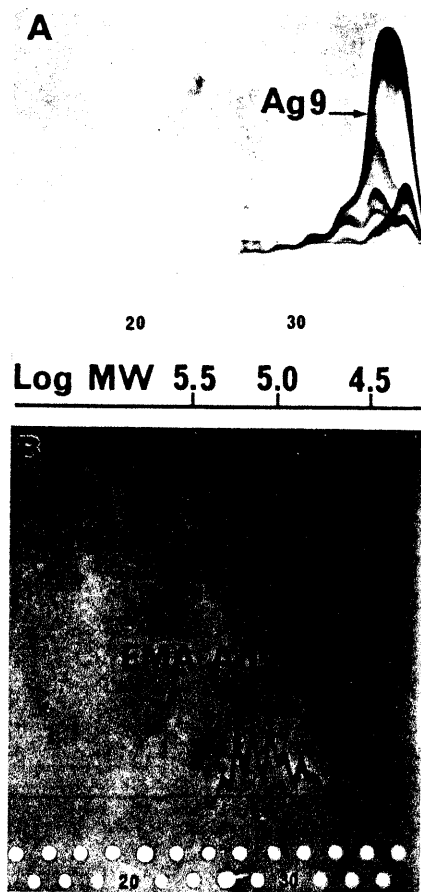


Fig. 1. Fused rocket immunoelectrophoresis. Samples gel-filtrated on Ultrogel ACA 22. Each well represents one fraction eluted from the column. Well numbers are indicated. Antibody: $8 \mu\text{l} \cdot \text{cm}^{-2}$ of rabbit anti-horse hair and dandruff. Molecular weights from eluted calibration antigens and the precipitates of Ag 9 and modified Ag 9 are indicated. Electrophoretic conditions: $2 \text{ V} \cdot \text{cm}^{-1}$ for 16 h at 15°C in 1% (w/v) agarose gel dissolved in 0.073 M Tris, 0.024 M barbital, 0.006 M calcium

To judge from reaction 3, where the mole-to-mole proportion between allergen and EMA was only 1:2, the efficacy of the coupling was rather high, and increase of EMA concentration did not increase the number of EMA chains attached. Furthermore, no correlation was observed between the number of available α - and ϵ -amino groups in the native allergens [Løwenstein *et al.*, 1976b] and the reaction products obtained. Comparison of the immunochemical elution profile after gel filtration of whole horse hair and dandruff versus EMA modification 2 is shown in figure 1. It might be suggested, though not yet verified, that all the products obtained were mixtures composed of one to several allergens and of one to several EMA chains, and that the chains might establish both intra- and intermolecular bridges. Thus, Ag 9 did not partially degrade to Ag 7 when denatured with guanidinium hydrochloride after previous modification with EMA as did the native allergen.

The activity of the modified allergens in relation to the native ones was reduced. Table III shows that the antigenic activity decreased by 75–99% as measured by SRID using rabbit antibodies against horse hair and dandruff. SRID (fig. 2) showed only one more or less diffuse precipitation ring, the enclosed area of which was taken as a measure of the preserved activity, whereas the higher resolving power of CIE and CLIE revealed several immunochemically

lactate, 0.003 M sodium azide ($\text{pH } 8.6$, 25°C). Anode uppermost. Dimension of antibody-containing gel: $100 \times 80 \times 1 \text{ mm}^3$. **A** 1 mg whole extract of horse hair and dandruff. **B** 0.75 mg modification 2 of Ag 9. The position as well as the jagged shape of the precipitate demonstrate the high molecular weight of the modified allergen.

Table III. Activity of modified allergens of horse hair and dandruff

No.	Allergen	Number of immunochemically distinct molecules ¹	Preserved activity of modified relative to native allergens		
			SRID, % ²	RAST inhibition, % ³	prick test, % ⁴
1	Ag 9	2	7	2	31 (0–48)
2	Ag 9	4	5	5	6 (0–25)
3	Ag 9	4	3	11	12 (0–10)
4	Ag 6	6	8	4	18 (0–50)
5	Ag 11	3	25	16	24 (0–44)
6	Ag 11	2	3	1,350	10 (0–27)
7	Ag 11	2	1	19	10 (0–30)

¹ Determined by comparison between the antibody reaction against native and modified allergens performed as CLIE.

² Mean values, each on the basis of 8 measurements.

³ Mean values, each on the basis of 4 serial measurements.

⁴ Mean values, each on the basis of 6 patients (No. 1–3 and 5–7) or 4 patients (No. 4); ranges given in parentheses.

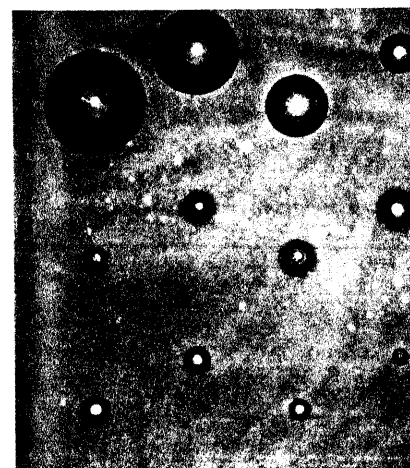


Fig. 2. SRID of Ag 11 (**A**), modification 5 of Ag 11 (**B**) and modification 6 of Ag 11 (**C**). Antibodies: $8 \mu\text{l} \cdot \text{cm}^{-2}$ of rabbit anti-horse hair and dandruff. Amounts of protein-antigen applied in the wells from left to right. **A** 14.0, 7.0, 3.0, 1.0 μg . **B** 1.6, 2.5, 4.9, 5.7 μg . **C** 27.0, 19.3, 11.6, 7.8 μg .

distinguishable molecules (fig. 3). The inhibitory effect of modified allergen in relation to the native one as measured by RAST was in all cases but one less than 20%; we have no explanation for the very high activity obtained by this method in modification 6. Finally, all the individual patients who were subjected to the prick test showed different degrees of reduced activity of the modified allergens. However, a considerable reduction of a patient's response to one modified allergen did not correspond with his response to another modification of the same allergen, thus demonstrating different reactivities against various allergenic sites of the molecules (not shown in table III). Furthermore, this different response to various allergenic sites differed from one patient to another. Immunochemical comparison between modified and native allergen by means of CLIE (fig. 3) revealed new precipitates containing modified antigens which were shown to be partially identical with the

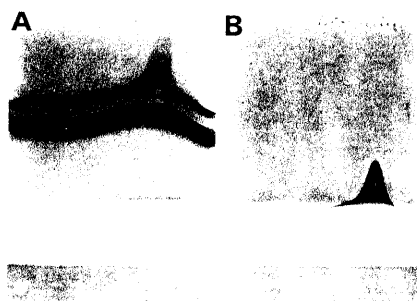


Fig. 3. CLIE. The various horizontal precipitation lines correspond to different modifications of Ag 6. Electrophoretic conditions: (1) dimension gel ($50 \times 10 \times 1.5$ mm³); $10 \text{ V} \cdot \text{cm}^{-1}$ for 25 min at 15°C ; (2) line gel ($50 \times 20 \times 1.2$ mm³), antibody-containing gel ($50 \times 40 \times 1$ mm³); $2 \text{ V} \cdot \text{cm}^{-1}$ for 16 h at 15°C . Buffer as described in figure 1. Anode: First dimension to the right; second dimension uppermost. **A** $0.5 \mu\text{g}$ Ag 6 applied in the well. **B** Control. $16 \mu\text{g}$ modified Ag 6 applied in the line gels of **A**.

native ones. This was interpreted as a direct or indirect effect of molecular shielding of attached EMA chains on the allergen. Thereby, the number and the nature of the antigenic sites are changed [Bock and Axelssen, 1973]. In this respect, each new precipitate developed represented a new combination of a reduced number of antigenic sites detectable with the antibodies used.

This study has demonstrated that by attaching flexible polyanionic chains to allergens, the activity was markedly reduced. Furthermore, patients' individual responses to unique allergenic sites have been indicated. These data have shown in all cases but one (modification 6), a decrease in activity caused by the actual modification: immunochromatological analysis on the basis of hyperimmunized rabbit IgG antibodies, IgE serum pool from selected patients and *in vivo* test

performed on allergic individuals demonstrated the reduced activity. The degree of preserved activity in relation to that of the native allergen was in all cases between 1 and 31% which was comparable with the results obtained by King *et al.* [1977] modifying AgE of ragweed by attaching chains of methoxypolyethylene glycol and dextran. Whether EMA-modified allergens might be useful for immunotherapy cannot be answered from these data, however.

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