

Comparison of IgE-binding antigens in horse dander and a mixture of horse hair and skin scrapings

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Extracts of horse dander (HD) and horse hair and skin scrapings (HHSS) have been compared with respect to their content of proteins and carbohydrates. The protein content of HD is more than double that of HHSS, while the carbohydrate content is of the same order. SDS–PAGE and IEF, both combined with immunoblotting, and CIE/CRIE showed the IgE-binding ability of the proteins/glycoproteins present in the two extracts. SDS–PAGE/immunoblotting showed the presence of mainly the same IgE-binding bands in the two extracts. Nine were detected in HD, and seven in HHSS. Four of these were glycoproteins. The most important allergen was a glycoprotein with molecular mass of 27 kDa showing IgE-binding ability to the 16 patient sera available. Another important allergen with molecular mass of 67 kDa is probably horse albumin. IEF/immunoblotting showed the presence of a glycoprotein with IgE-binding ability at pH 3.2 in both extracts. Tandem CRIE showed that both extracts contained the same four allergens as major allergens. In addition, as we observed when analyzing both extracts in CRIE against 16 different patient sera, each extract contained allergens not present in the other extract. We may conclude from this investigation that commercial allergen extracts to be used in diagnosis and immunotherapy should include both horse dander and horse hair and skin scrapings in the starting material for the preparation of extracts. If not, allergens which may be of importance for some patients will be missing.

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Hypersensitivity to animal dander or serum is a well-known phenomenon. This situation has been reviewed by Squire (21), who also, as early as 1950, showed that horse serum albumin is an allergen. Later Stanworth (22, 23) isolated an allergen from horse dander by physicochemical separation methods combined with immunochemical identification. Ponterius et al. (20) found, by a combination of PAGE and RAST, horse albumin and two other proteins in horse dander that show allergenic activity. A partial separation of horse-epithelium extract by Aronsson & Wide (1) showed the presence of several allergens. The antigenic components in extracts of horse hair and dandruff have been characterized and the allergens identified by CRIE. Löwenstein et al. (10, 11) and Markussen et al. (12) isolated and partially characterized four allergens from horse hair and dandruff. Recently, Franke et al. compared five different horse epithelial allergen extracts by biochemical and

immunochemical methods, finding similarities, but also marked differences, among the extracts (5). The present study was undertaken to compare the protein/glycoprotein content in extracts of horse dander and of horse hair and skin scrapings, to evaluate their ability to act as antigens and allergens by SDS–PAGE and isoelectric focusing (IEF) combined with immunoblotting and CIE/CRIE, and to identify possible allergens in horse dander and in hair and skin scraping extracts which may be of importance for diagnosis of horse allergy.

Material and methods

Materials

Allergen extracts. Horse dander (batch 204683004) and powdered horse hair and skin scrapings (batch 204381006) were obtained from Allergen AB, Engelholm, Sweden. Freeze-dried extracts were made

from the raw materials by the following procedure: 1:20 w/v of each powder was extracted with 0.125 M ammonium bicarbonate buffer, pH 7.95, at 4°C for 20 h, followed by centrifugation (13 000 rpm), filtration, and freeze-drying. The extract prepared from horse dander is referred to as HD, and that from horse hair and skin scrapings as HHSS. The extracts were dissolved in 2% *n*-butanol in water to a concentration of 4 mg/ml of HHSS and 40 mg/ml of HD, transferred to vials, and freeze-dried. The vials, containing approximately 2 mg or 20 mg HHSS, and 20 mg or 200 mg HD, were stored at -20°C.

Human sera. Sera from 16 patients between 15 and 73 years of age were provided by Nycomed Pharma AS. All patients had positive skin tests for horse allergens and a history of allergy to horses. The RAST classes of the sera were as follows: 3 and higher – three sera; between 1 and 3 – seven sera; RAST class 0 – six sera. Six sera were from men, and the remaining 10 from women. Serum from a nonallergic woman and a serum pool prepared from mugwort allergics (RAST class 3 and higher) were used as control sera (17). The sera were stored at -20°C.

Rabbit antiserum. Rabbit anti-horse-dander serum was kindly provided by Pharmacia AB (L.J. 327569 841212 SP). It was stored at -20°C.

Methods

Protein/carbohydrate content

Total content of protein in the extracts was measured by the modified method of Lowry (3) with bovine serum albumin as reference. Carbohydrate content was determined by gas chromatography of the TMS derivatives of the methylglycosides obtained by methanolysis of the extracts (4).

SDS-PAGE

Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate in a Mini-Protean II dual slab cell (14) by Laemmli's discontinuous buffer system (9). Gels (0.75 mm thick) containing, respectively, 4% (stacking gel) and 12% (separating gel) acrylamide were prepared with a total monomer concentration (%T) of 30, and a cross-linking monomer concentration (%C) of 2.76.

Freeze-dried extracts were dissolved in distilled water. Equal amounts of sample solution and sample buffer were heated to 95°C for 4 min. The final concentration in sample buffer was then that of Laemmli (9). Electrophoresis was performed with a constant voltage (200 V) for approximately 50 min. From each extract a sample of 100–200 µg was dissolved, as

mentioned above, and applied in the wells. The Low Molecular Weight Calibration Kit (Pharmacia, Uppsala, Sweden) was used as molecular weight marker. The gels were stained with Coomassie brilliant blue (CBB) R-250 to detect proteins, and with the periodate-Schiff reagent (PAS staining) to detect carbohydrate polymers (25). Densitometric scanning of the CBB- and PAS-stained gels was performed at 550 nm with the Shimadzu (Kyoto, Japan) CS-9000 dual wavelength flying-spot scanner.

Immunoblotting of SDS-PAGE gels and detection of IgE-binding antigens

After separation of the samples by SDS-PAGE, the proteins/glycoproteins were transferred to nitrocellulose membranes (NCM) with the Mini-Trans Blot Cell from Bio-Rad (15). The buffer used contained 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3 (15). The transfer time was 1 h at 100 V. Unoccupied sites were blocked by a buffer consisting of 10% v/v 50 mM phosphate buffer, pH 7.4; 0.9 w/v sodium chloride; 0.3% w/v dextran 70; and Tween 20 0.5% w/v. Blocking time was 1 h at 37°C.

The membranes were then incubated overnight with patient serum, diluted 1:3 with blocking buffer. IgE-binding antigens were detected after incubation with ¹²⁵I-labeled anti-IgE overnight, followed by autoradiography for periods varying from 3 h to 3 weeks.

Isoelectric focusing (IEF)

Analytic IEF in the pH range 3–10 was performed in polyacrylamide gels made as previously described (17). IEF was performed at constant power (25 W) for 1.5 h.

The samples were prepared by dissolving the freeze-dried extracts in distilled water (7). The amounts applied were equivalent to 100–300 µg of both extracts.

Immunoblotting of IEF gels and detection of IgE-binding antigens

Transfer of the separated compounds to NCM was performed in 0.7% acetic acid buffer for 5 h with a constant voltage of 70 V in a Trans-Blot Cell from Bio-Rad, according to the manufacturer's instructions. Further immunologic procedures were as described above under immunoblotting of SDS-PAGE gels.

CIE/CRIE

Crossed immuno- and radioimmuno-electrophoresis (CIE/CRIE) was performed in gels made with Bio-

Rad's agarose immunoelectrophoresis tablets, as described by Høiby & Axelsen (6), with a Tris-Tricine buffer, pH 8.6. The samples applied consisted of aqueous solutions of freeze-dried extracts equivalent to 16 µg HD and 75 µg HHSS. The first-dimension electrophoresis was run for 40 min at 10 V/cm. The second-dimension electrophoresis was run perpendicularly to the first in an agarose gel containing 10 µl of rabbit anti-horse-dander antibody per cm² gel. The electrophoresis was performed for 20 h at 2 V/cm. The gels were then washed in the usual way and dried in cold air.

Detection of IgE-binding antigens

Unstained CIE plates were incubated overnight with patient serum, diluted with CRIE buffer (0.9% w/v sodium chloride; 0.3% w/v dextran 70; 0.3% v/v Tween 20; and 10% v/v phosphate buffer pH 7.4), followed by incubation overnight with ¹²⁵I-labeled anti-IgE, diluted with CRIE buffer. After incubation with patient serum, the plates were washed as previously described (18). Incubation with ¹²⁵I-labeled anti-IgE overnight was followed by 4 × 30-min washings with CRIE washing-solution, and 1 × 30-min washing with distilled water and air drying. The plates were then subjected to autoradiography for up to 9 days.

Tandem CIE/CRIE was performed under equal conditions, but this time the second-dimension gel contained 2.5 µl antiserum per cm².

Results

Raw material and content of the extracts

As shown by Table 1, the total yield of the freeze-dried extract from the raw material of horse dander was about 10 times as high as that from horse hair and skin scrapings. Furthermore, the protein con-

tent of the extracts, as determined by the modified Lowry method, showed the HD extract to contain 35% protein, while the HHSS extract contained 14%. Table 1 shows that HD contained 8% and HHSS 11% carbohydrate. The carbohydrate material of both extracts consisted of mannose, galactose, glucose, *N*-acetylglucosamin, and sialic acid, the amount of glucose being distinctly higher in HHSS than in HD.

SDS-PAGE/immunoblotting

Separation by SDS-PAGE, followed by staining with CBB to detect proteins and PAS staining to detect carbohydrate polymers, showed great similarities between the two extracts. In the molecular mass range between 14 and 94 kDa, 12 protein bands were detected in the HD extract and eight in HHSS (Fig. 1). Polymers of similar molecular masses were found to be present in both extracts. PAS staining revealed the presence of four glycoproteins (Fig. 1), all of which proved to be important IgE-binding antigens (Table 2).

Immunoblotting, followed by incubation with patient sera and autoradiography, showed that all sera available had IgE-binding capacity towards a glycoprotein with molecular mass of 27 kDa (Fig. 1, band 7; Table 2). The remaining glycoproteins, band 2, molecular mass 14.5 kDa; band 5, 20 kDa; and band 8, 31 kDa, as well as band 11, 67 kDa, all appeared to be important allergens. No radiostaining was observed when the NCM blots were incubated with serum from a nonallergic person or with a serum pool from mugwort-allergic patients.

Densitometric scanning of the CBB- and PAS-stained gels of the HD extract revealed four distinct protein and carbohydrate peaks corresponding to band numbers 2, 5, 7, and 8 in the gels. In the HHSS

Table 1. Yields and chemical characterization of the raw extracts prepared from horse dander (HD) and horse hair and skin scrapings (HHSS)

	HD	HHSS
Yield from raw material (weight)	36.6%	3.1%
Protein content in extracts ^a	35.0%	14.0%
Total carbohydrate content ^b in the extracts	8.0%	11.0%
	% of total carbohydrate	
Mannose	11.6%	1.2%
Galactose	9.1%	2.6%
Glucose	32.3%	74.3%
<i>N</i> -acetylglucosamin	32.0%	12.6%
Sialic acid	15.0%	9.2%

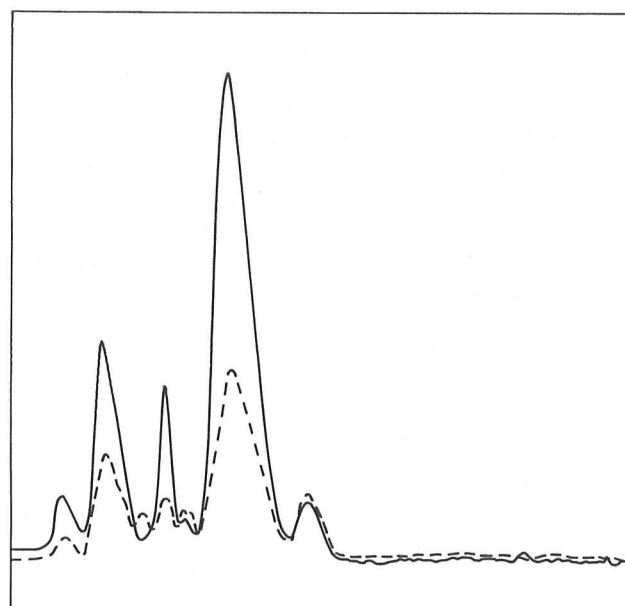
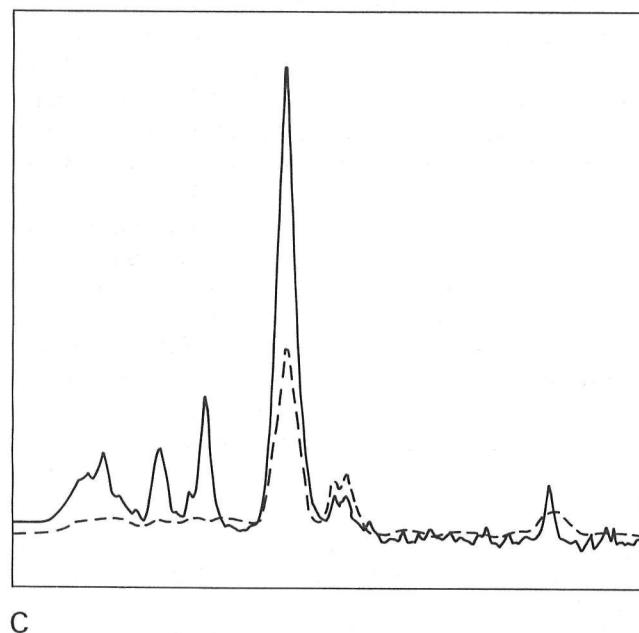
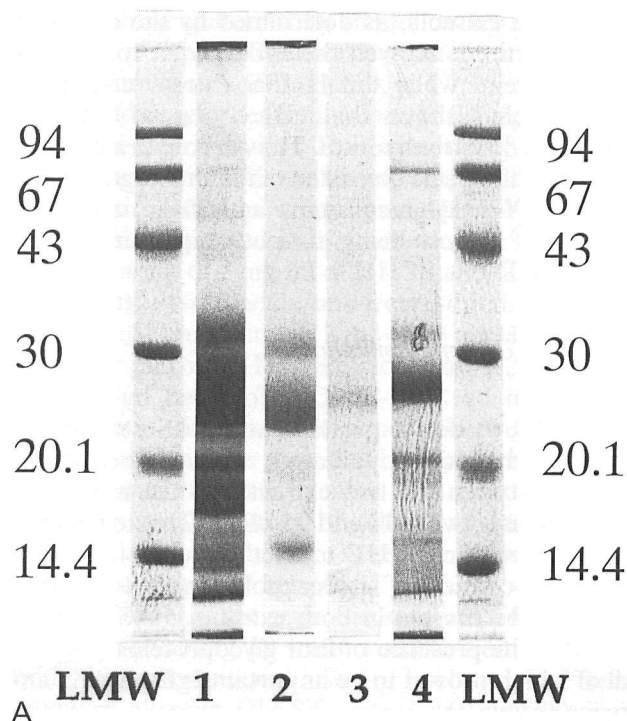
^a Determined by the modified Lowry method.

^b Determined by GC as the TMS-derivative of the methyl glycosides.

Table 2. Number of patient sera showing IgE-binding ability towards protein/glycoprotein bands in the HD and HHSS extracts after separation by SDS-PAGE, immunoblotting, and autoradiography

Mol. mass (kDa)	Protein band number	HD extract	HHSS extract
14	1	0	np
14.5	2 (glycoprotein)	8	5
16	3	0	np
17	4	3	0
20	5 (glycoprotein)	6	6
24	6	0	np
27	7 (glycoprotein)	16	16
31	8 (glycoprotein)	12	9
42	9	3	1
50	10	4	3
67	11	9	7
94	12	1	np

np: not present.



B

Fig. 1. A) Horse epithelial extracts (HD and HHSS) analyzed by SDS-PAGE and stained with CBB to detect protein and with PAS to detect carbohydrate. Lanes marked LMW are molecular mass markers; lanes 1 and 4 are CBB-stained gels of extracts HD and HHSS, respectively; lanes 2 and 3 are PAS-stained gels of the same extracts. B) and C) are scans of HD and HHSS SDS-PAGE gels. Protein ———. Carbohydrate - - - -.

gels, a similar pattern was found for the protein content, whereas there was a marked difference concerning the carbohydrate content. The HHSS gels showed similarity with the HD gels in the peaks corresponding to band numbers 7 and 8, while there were hardly any peaks in the HHSS gels corresponding to band numbers 2 and 5 (Fig. 1).

IEF/immunoblotting

Analytic IEF performed in a pH-gradient between 3 and 10, showed the HD extract to have protein bands

in the area pH 3–4.5, while protein bands from the HHSS extract were found between pH 3.5 and 5.5. Detection of glycoproteins by PAS staining visualized two distinct bands at pH 3.2 and 3.5, respectively, in both extracts. Two more bands were barely visible in the area pH 4–5.

Immunoblotting, incubation with patient sera, and autoradiography showed IgE-binding to a glycoprotein with pI 3.2 in both extracts. Additionally, three IgE-binding bands were observed in the HD extract, one of which was also present in the HHSS extract. Five of the six RAST class 0 sera gave hardly any

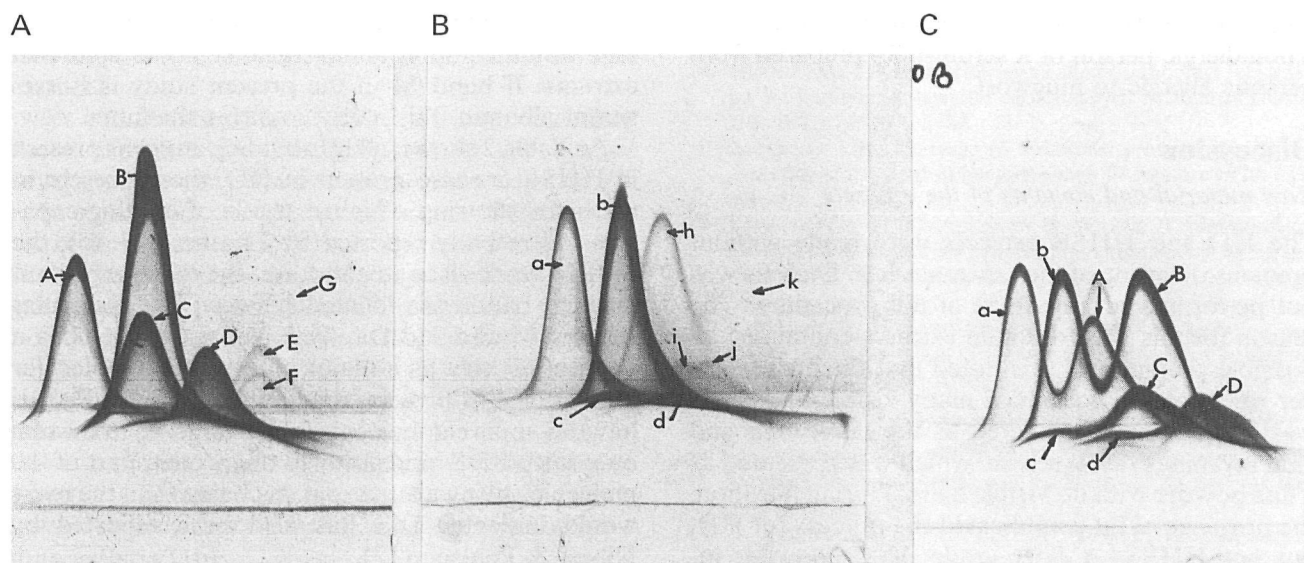


Fig. 2. Crossed immunoelectrophoresis (CIE) of A) horse dander extract; B) horse hair and skin scrapings extract; C) tandem-CIE of the two extracts.

reaction in the immunoblotting experiments performed on the IEF gel.

CIE/CRIE

By using rabbit anti-horse-dander serum in the second-dimension gels, horse-dander-specific antigens could be detected in the two different horse epithelial extracts. Staining with CBB showed seven precipitates from the HD extract, while eight were detectable in the HHSS extract (Fig. 2). Four of these precipitates, designated A, B, C, and D in HD, and corresponding precipitates, designated a, b, c,

and d in HHSS, were present in both extracts, as confirmed by tandem CIE (Fig. 2). No dander-specific, cathodic, migrating antigens were found.

CRIE analysis of both extracts was performed for each of the 16 patient sera available. Table 3 shows that the four precipitates designated A, B, C, and D and a, b, c, and d, respectively, were present in both extracts as major allergens; that is, they showed binding to at least 50% of the patient sera used (2). It should be noted that precipitates h and k were present only in the HHSS extract and showed allergenic activity towards four and three, respectively, of the patient sera. No radiostaining was observed

Table 3. The presence of IgE in individual patient sera against antigens in extracts from horse dander and horse hair and skin scrapings determined by CRIE

Patient no.	Horse dander (HD) precipitates							Horse hair and skin scraping (HHSS) precipitates							
	A	B	C	D	E	F	G	a	b	c	d	h	i	j	k
1	X	X	X	X				X	X	X	X				
2	X	X	X	X	X			X	X	X	X				
3	X	X	X	X			X	X	X	X	X				X
4	X	X	X	X				X	X	X	X				
5	X	X	X	X				X	X	X	X	X			X
6	X	X	X	X				X	X	X	X				
7	X	X	X	X				X	X		X				
8	X	X	X	X				X	X		X				
9	X	X	X	X				X	X		X	X			
10	X	X	X	X			X	X	X		X				X
11	X	X	X	X				X	X		X				
12	X	X	X	X	X			X	X		X	X			
13	X	X	X	X	X			X	X	X	X	X			
14	X	X	X	X				X	X		X				
15	X	X	X	X				X	X	X	X				
16	X	X	X	X	X			X	X		X				
Sum	16	16	16	16	4	0	2	16	16	8	16	4	0	0	3

when the CIE plates were incubated with serum from a nonallergic person or a serum-pool prepared from persons allergic to mugwort.

Discussion

Raw material and contents of the extracts

The HD and HHSS extracts were made without previous defatting of the raw materials. Dialysis was not performed at any stage of the procedure. The reason for this was to obtain extracts containing all possible allergens. It was noted that the horse dander raw material contained many visible hairs, and was not as finely powdered as the horse hair and skin scrapings raw material, which was presented as a fine powder with no visible hairs. During filtration, the presence of fat complicated the process for HD, but not HHSS. A finer grade of the powder increases the solubility of fat.

The high glucose content may be due to traces of serum and also to the content of horse hair. It has been shown that animal hair, such as cat (8), dog (19), and also human (13), contains considerable amounts of glucose, as compared to the other sugars present. This might also account for the somewhat higher percentage of total carbohydrate present in HHSS.

As the freeze-dried extracts were prepared without dialysis, there is reason to assume the presence of salts and low-molecular-mass compounds as well as fatty components and emulsifiers, factors which could lead to the low content of protein and carbohydrate of the extracts.

SDS-PAGE/immunoblotting

As approximately the same amount of extract by weight was applied in the wells, it can be noted from Fig. 1 that the bands from HHSS are weaker than those from the HD extract. This corresponds well with the findings of total protein content in the extracts (Table 1), where HD was found to contain more than twice as much protein as HHSS. Throughout the experimental procedures, the HD extract seemed to be much more concentrated in its antigen content than the HHSS extract, apart from band 11 with molecular mass 67 kDa. The presence of this protein further confirms the presence of traces of serum components in the raw material.

Allergens giving an IgE-positive reaction in more than 50% of the patient sera tested are often classified as major allergens (2). Previous investigators of horse epithelial extracts have advanced different views on the importance of serum albumin as an allergen. While Løwenstein et al. (10), Wahn et al. (24), and Franke et al. (5) all found horse serum albumin to be of minor allergenic importance, Squire

(21) and Ponterius et al. (20) concluded that albumin was an important allergen in horse epithelial extracts. If band 11 in the present study is horse-serum albumin, this study confirms the latter view.

As Table 2 shows, all IgE-binding antigens present in HHSS are also present in HD, those present in the latter showing a higher degree of binding capacity. As recently reported by Franke et al. (5), the present work also indicates that the most important allergen bands are located between the molecular masses 14 and 31 kDa. This study also includes a band of 67 kDa as a major allergen. The molecular masses of glycoproteins determined by SDS-PAGE have an apparent molecular mass larger than the real one, since SDS binds only to the protein part of the molecule, giving a less negative charge than the mass would indicate. This has also been reported by Nilsen & Paulsen (17).

It was interesting to note that only one of the RAST class 0 sera showed a very weak IgE binding in the SDS-PAGE immunoblotting experiments; the other five appeared to give comparable intensity with the RAST-positive sera. Neither the nonallergic nor the mugwort serum-pool gave positive radiostaining when these were used as controls.

IEF/immunoblotting

The results from IEF correspond well to those obtained by Løwenstein et al., who found IgE-binding components in a pH range of 3.5–4.5 (11). What differs in the present study is the detection of a glycoprotein with IgE-binding ability at pH 3.2 present in both extracts. IEF showed that all allergens detected by this method in the HHSS extract were also found in the HD extract.

CIE/CRIE

CRIE analysis was performed for both extracts for each of the 16 patient sera available, in addition to serum from a nonallergic person and a mugwort-allergic serum-pool. Table 3 shows that the four precipitates designated A, B, C, and D and a, b, c, and d, respectively, were present in both extracts as major allergens; that is, they showed binding to at least 50% of the patient sera used (2). Tandem CRIE showed that these four allergens in each extract were immunochemically identical (Fig. 2). It should be noted that precipitates h and k were present only in the HHSS extract and showed allergenic activity for four and three, respectively, of the patient sera. The presence of these antigens in HHSS is a reason to include horse hair and skin scrapings as well as horse dander in the raw material for production of extracts for diagnosis and immunotherapy for horse allergy. They were not present in detectable amounts

in the horse dander extract, which also contained two allergens, E and G, not present in the HHSS extract.

As can be seen from Table 3, all sera, including those with RAST class 0, contained IgE against several of the antigens present in both extracts.

In conclusion, it is interesting to note that although six of the skin-prick-positive sera had RAST class 0, the three different methods used for testing allergenicity in this paper gave different results. All gave positive CRIE pictures, and only one gave a very weak reaction in the SDS-PAGE/immunoblotting system, while five gave negative reactions with IEF/immunoblotting. These results show that none of these methods can be used alone to determine whether a patient is allergic to horses. Such results have also been found for the diagnosis of house-dust mite asthma (16).

Further studies are in progress to isolate and characterize the glycoprotein-allergens identified.

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