

Allergoprints in Horse Allergic Children

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In order to determine the allergenic activity of five purified horse allergens, 22 children allergic to horses according to history, skin test, and leukocyte histamine release were evaluated. Washed leukocytes from all patients were tested for allergen-induced histamine release utilizing four epidermal horse allergens (Ags 6, 9, 11, and 15) and horse serum albumin. Crossed radioimmunoelectrophoresis was carried out with a standardized horse dander extract and serum from each patient.

The results showed considerable variation in the individual allergoprints. Ag 11 had the highest mean allergenic activity. Sensitivity to horse serum albumin was demonstrated three times. Our data show that the amount of serum IgE antibodies bound by horse allergens correlates significantly with the capacity of the allergens to induce histamine release from washed leukocytes.

Key words: allergens; allergy to animals; crossed radioimmunoelectrophoresis; histamine release.

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CLINICAL ASPECTS

Horse allergic individuals are sensitized to different spectra of allergenic proteins, which is demonstrated by CRIE and histamine release from leukocytes. The majority of patients are allergic to the four epidermal allergens (Ags 6, 9, 11 and 15), while horse serum albumin is a minor allergen. Extracts used for hyposensitization treatment should include all major allergens. Information should be available on the content of allergenic molecules in an extract.

Animals, like other allergen sources, are multi-allergenic systems. An allergen extract of horse hair and dander has previously been shown to contain 21 different antigens detectable by crossed immunoelectrophoresis (CIE) (7). Many of these proteins are able to bind IgE-antibodies from the serum of horse allergic individuals as demonstrated by means of crossed radioimmunoelectrophoresis (CRIE) (3), although there was a great variability in patients' individual response, both in strength of IgE binding and allergen specificity of their IgE.

Based upon such CRIE analyses the generally most important allergens, Ags 6, 9 and 11, could be recognized and were subsequently isolated. Their allergenic activities were verified by means of RAST inhibition and skin prick test, and a physico-chemical characterization was performed (4, 5). Recently, one further

epithelial allergen (Ag 15) has been isolated and physico-chemically characterized (2).

In order to study hypersensitivity to horses on a molecular level, these four allergens as well as horse serum albumin were used to trigger the basophils of horse allergic children for IgE-mediated histamine release. The histamine releasing activity of these allergens towards the patients' leukocytes was compared with the ability of these allergens to bind IgE antibodies from the patients' sera.

MATERIAL AND METHODS

Patients. The study comprised 22 children (13 female, 9 male) aged 2-17 years, who according to their history, skin prick test and leukocyte histamine release were found to be allergic to horses. All had developed rhinoconjunctivitis

Table 1
Horse allergens

Antigen No.	Mol.wt.* ($\times 10^3$)	pI**	Carbohydrate (%)	Miscellaneous	Reference
6	19	4.1		amino acid composition	4
9	51	3.83 4.09	5	amino acid composition two peptide chains strongly hydrophilic	2, 4, 6
11	31	3.9	10	amino acid composition	4, 6
15	36	3.75			2
Serum-Albumin	68	4.5			3

* Determined by gel filtration on Ultrogel[®] ACA 54 or Sephadex G-75.

** Determined by IEF on sucrose gradient column or on polyacryl gel.

Table 2
Score system in CRIE*

Days of autoradio- graphy		Pool of sera					
		1	2	3	5	7	10
Individual sera	10	1	2	3	5	7	10
	3	3	6	9	15	21	30
	1	10	20	30	50	70	100

* Numbers in table give the CRIE scores. Use of table: If a precipitate is radioactive after 1 day of autoradiography to a degree corresponding to 7 days with the serum pool, then the scores will be $10 \times 7 = 70$.

All data on Ag 11 are corrected for non-specific IgE binding corresponding to score 1 after 10 days.

and/or asthmatic symptoms on exposure to horses.

Allergenic extracts. The horse allergen extract (Soluprick[®] SQ, ALK, Copenhagen, Denmark) used for skin prick test and histamine release had been prepared from hair and dander and was standardized for content of individual allergens and total allergenic activity by means of CIE/CRIE and RAST inhibition. Its activity was given in HEP units (1).

Individual allergens. Four epidermal horse allergens (Ags 6, 9, 11 and 15) were used for histamine release experiments. The isolation and physico-chemical characterization has been described (2, 4, 5), and the main parameters appear from Table 1. Horse serum albumin was obtained commercially from SIGMA Chem. Co., St. Louis, Mo., U.S.A.

Solutions. A pipes buffered medium (pH 7.4) was used, 1 l containing 110 mmol calcium chloride, 5 mmol potassium chloride, 40 mmol sodium hydroxide, 25 mmol Pipes and 300 mg human serum albumin (Pipes A). To this medium 2 mmol calcium chloride and 0.5 mmol magnesium chloride were added to prepare Pipes ACM used for diluting antigen and cells for the histamine release reaction.

Preparation of leukocytes. Blood from all patients was collected in plastic syringes and anticoagulated with 0.01 M EDTA. It was sedimented at room temperature for 60–75 min with 2.5 ml dextran (clinical dextran 6% (w/v) in 0.85% NaCl (w/v), to which 3% (w/v) glucose was added) per 10 ml blood. The upper layer containing leukocytes, platelets and plasma was transferred to another plastic tube and centrifuged at 300 g for 8 min at 4°C. The pelleted cells

Table 3

Allergenic activity of horse antigens, expressed as the antigen concentration necessary for 30% histamine release

Patient No.	Horse extract (HEP)	Ag 6 (M)	Ag 9 (M)	Ag 11 (M)	Ag 15 (M)	Horse alb. (M)
1. P.P.	$3 \cdot 10^{-6}$	$1.9 \cdot 10^{-11}$	$5.2 \cdot 10^{-11}$	$5.7 \cdot 10^{-12}$	$1.8 \cdot 10^{-11}$	—+
2. M.H.	$2.3 \cdot 10^{-6}$	$9 \cdot 10^{-11}$	$5.3 \cdot 10^{-12}$	$97 \cdot 10^{-11}$	$1.3 \cdot 10^{-11}$	—+
3. I.H.	N.D.	$6.5 \cdot 10^{-12}$	$8 \cdot 10^{-13}$	$2.6 \cdot 10^{-13}$	$4.2 \cdot 10^{-12}$	+
4. W.J.	$2.2 \cdot 10^{-6}$	$1.7 \cdot 10^{-10}$	$5 \cdot 10^{-10}$	$3.9 \cdot 10^{-12}$	$1.3 \cdot 10^{-11}$	—+
5. L.A.	$2.4 \cdot 10^{-5}$	$2.1 \cdot 10^{-9}$	$7.5 \cdot 10^{-11}$	$5.5 \cdot 10^{-11}$	—****	—+
6. C.S.	$2.4 \cdot 10^{-6}$	$1.8 \cdot 10^{-11}$	$8.5 \cdot 10^{-12}$	$1 \cdot 10^{-11}$	$1.2 \cdot 10^{-8}$	—+
7. T.L.	$1.2 \cdot 10^{-6}$	$1.2 \cdot 10^{-11}$	$8.5 \cdot 10^{-12}$	$2 \cdot 10^{-12}$	$2.6 \cdot 10^{-11}$	—+
8. M.H.	$6 \cdot 10^{-6}$	$9.5 \cdot 10^{-11}$	$3.8 \cdot 10^{-11}$	$4.2 \cdot 10^{-12}$	$2 \cdot 10^{-11}$	—+
9. Y.H.	$2.2 \cdot 10^{-5}$	$1.6 \cdot 10^{-9}$	$9 \cdot 10^{-10}$	$1 \cdot 10^{-10}$	$1.1 \cdot 10^{-10}$	—+
10. A.S.	$7.5 \cdot 10^{-7}$	$1.9 \cdot 10^{-10}$	$7.5 \cdot 10^{-11}$	$1 \cdot 10^{-12}$	—****	—+
11. T.R.	$1.8 \cdot 10^{-4}$	$1.1 \cdot 10^{-7}$	$3 \cdot 10011-10$	$1.4 \cdot 10^{-10}$	$3.8 \cdot 10^{-11}$	$5.5 \cdot 10^{-8}$
12. P.W.	$1 \cdot 10^{-8}$	$6 \cdot 10^{-13}$	$2 \cdot 10^{-12}$	$3 \cdot 10^{-9}$	$6 \cdot 10^{-14}$	$1 \cdot 10^{-8}$
13. P.C.	$7.5 \cdot 10^{-6}$	$3.8 \cdot 10^{-10}$	$1.7 \cdot 10^{-10}$	$3.8 \cdot 10^{-11}$	$3.4 \cdot 10^{-8}$	—+
14. M.S.	$3 \cdot 10^{-3}$	$2.6 \cdot 10^{-9}$	$2.4 \cdot 10^{-9}$	$5 \cdot 10^{-9}$	$2.2 \cdot 10^{-9}$	—+
15. R.H.	$1.5 \cdot 10^{-6}$	$1 \cdot 10^{-10}$	$3.2 \cdot 10^{-11}$	$2.5 \cdot 10^{-12}$	$7 \cdot 10^{-12}$	—+
16. D.R.	$1.1 \cdot 10^{-4}$	$1.4 \cdot 10^{-9}$	$5.4 \cdot 10^{-10}$	$2.5 \cdot 10^{-11}$	—****	—+
17. G.W.	$3.8 \cdot 10^{-7}$	$3.4 \cdot 10^{-12}$	$5 \cdot 10^{-12}$	$8 \cdot 10^{-13}$	$5.5 \cdot 10^{-11}$	$5 \cdot 10^{-11}$
18. S.S.	$4.4 \cdot 10^{-4}$	$8.5 \cdot 10^{-8}$	$6.5 \cdot 10^{-9}$	$2.8 \cdot 10^{-10}$	—****	—+
19. D.F.	$2.1 \cdot 10^{-3}$	—***	$8.5 \cdot 10^{-11}$ *	$1.6 \cdot 10^{-8}$ *	—****	—+
20. H.S.	$1.2 \cdot 10^{-5}$	$1.3 \cdot 10^{-9}$	$6 \cdot 10^{-10}$	$4.4 \cdot 10^{-11}$	$1 \cdot 10^{-}$	—+
21. K.U.	$5.25 \cdot 10^{-4}$	$6.5 \cdot 10^{-9}$ *	$5.2 \cdot 10^{-8}$ *	$3.6 \cdot 10^{-8}$ *	$2.2 \cdot 10^{-9}$ *	—+
22. M.S.	$1 \cdot 10^{-4}$	$3.4 \cdot 10^{-9}$	$1.2 \cdot 10^{-9}$	$1.2 \cdot 10^{-10}$	—****	—+

N.D. = not done.

* = allergen concentration for 20% histamine release.

** = allergen concentration for 50% histamine release.

*** = no release up to 7×10^{-8} M.**** = no release up to 2×10^{-8} M.+ = no release up to 4×10^{-8} M.

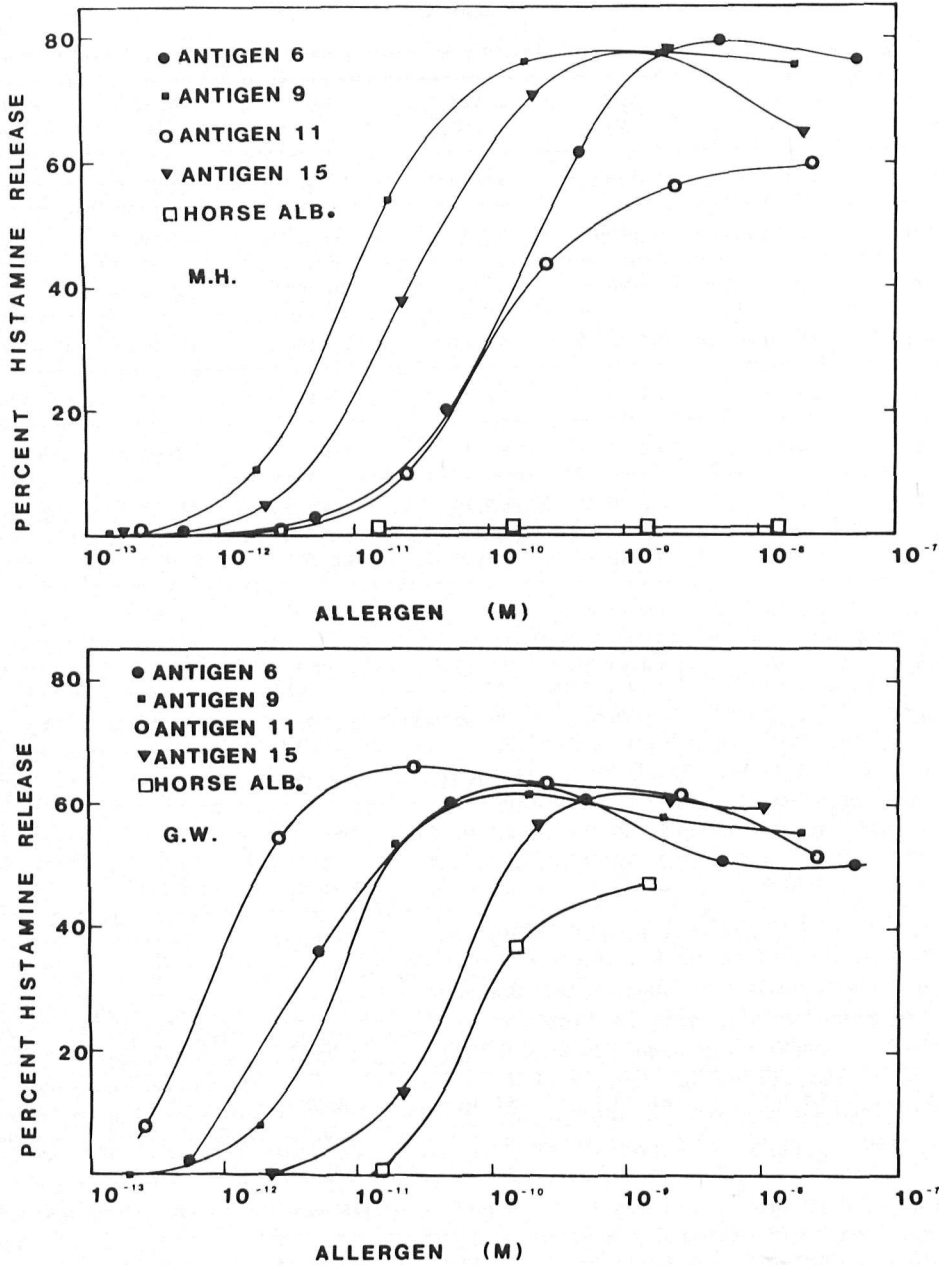


Fig. 1. Dose response curve for allergen-induced histamine release from leukocytes of two horse-sensitive individuals, using four epidermal horse antigens and horse serum albumin.

were suspended in Pipes A containing 4 mmol EDTA, and centrifuged at 300 g for 8 min at 4°C. The cells were then washed in Pipes A. After centrifugation the cells were suspended in Pipes ACM-buffer.

Histamine release was carried out at 37°C for 40 min. In all experiments reaction tubes were in duplicate. Blanks contained cells and buffer. Histamine release in these tubes did not exceed 10% of the completes. Cellular histamine con-

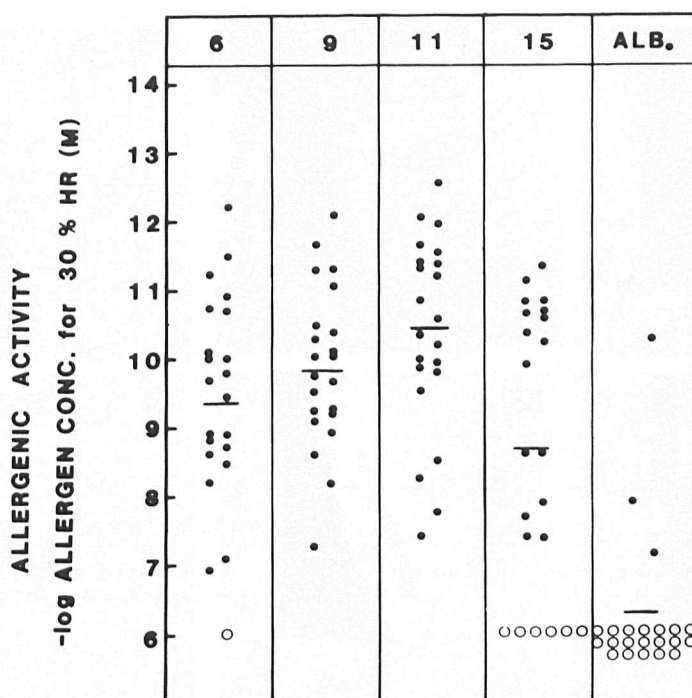


Fig. 2. Summary of allergenic activities of four epidermal horse antigens and horse serum albumin in 22 horse-sensitive individuals. Open circles indicate that there was less than 30% histamine release at allergen concentrations up to 10^{-6} M.

tent for each experiment (completes) was determined by perchloride acid lysis of cells.

Histamine assay. Histamine was assayed by the automated fluorometric technique described by Siraganian (8, 9). Calculations were as described previously (10).

The allergenic activity of an allergen as determined by the histamine release assay was expressed as the molar allergen concentration which was necessary for 30% histamine release in the ascending part of the dose response curve.

Crossed radioimmuno-electrophoresis. Serum samples from all children were taken together with the leukocytes and kept frozen at -20°C until used for CRIE. CRIE was carried out as previously described (6, pp. 23–24 and 31–37) using experimental conditions for the CRIE system as described in Fig. 3. CRIE data were expressed by the score system described in

Table 2. A serum pool from 1,000 normal individuals was used as a negative control.

Statistics. The means were calculated using a Wang 2200 calculator. Correlation analysis was done by Spearman's rank correlation tests.

RESULTS

All 22 patients who developed allergic symptoms on exposure to horses had positive skin tests with our partly purified and standardized allergen extract from horse hair and dander, and their leukocytes released histamine on challenge with the same extract. The concentration of this extract required for 30% histamine release varied between 1×10^{-8} HEP and 3×10^{-3} HEP.

A summary of the histamine release data from all patients studied is presented in Table 3. All the children showed a significant histamine release on exposure to Ags 9 and 11

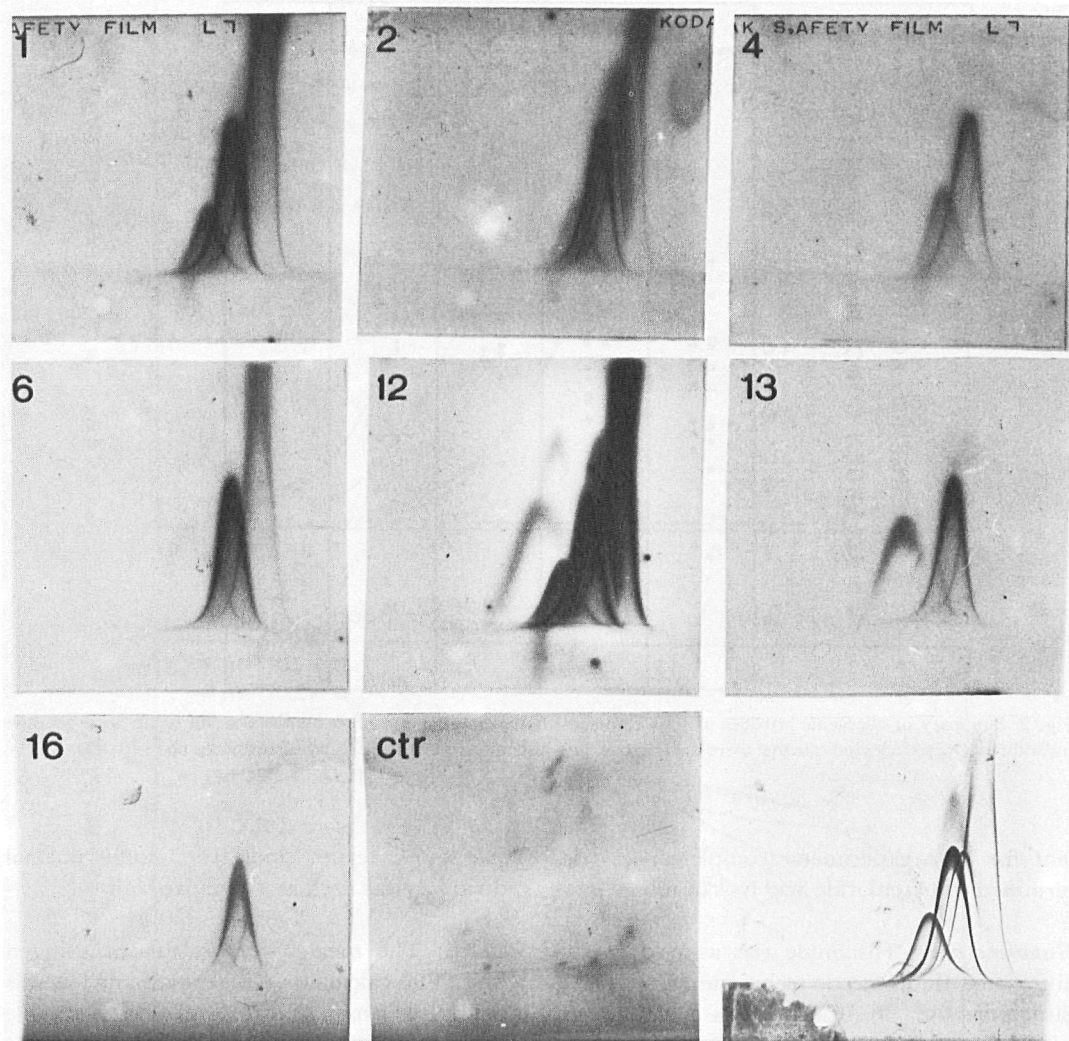


Fig. 3. Allergoprints for seven horse dander-allergic patients (numbers indicate patient identity), pool of sera from 1,000 normal individuals (control) together with the CRIE standard reference plate with identified important allergens (bottom right). CRIE was carried out as described recently (6), using 7.5 μ g horse dander extract as antigen and 15 μ l anti-horse dander per cm^2 as antibodies. Crossed immunoelectrophoresis in 1% (w/v) agarose gel containing 0.073 M TRIS, 0.024 M barbital and 0.0006 M calcium lactate (pH 8.6, 25°C) at 15°C; first dimension electrophoresis: 10 volt cm^{-1} in a $10 \times 50 \times 1.5$ mm³ gel for 25 min; anode to the right; second dimension electrophoresis: 2 volt cm^{-1} for 15 h into the antibody-containing gel ($40 \times 50 \times 1.0$ mm³).

Sera from the seven patients and the control were added to the "native crossed immunoplates". Autoradiography was carried out for 10 days.

(two of them less than 30%, but more than 20%); there was no histamine release at the tested allergen concentration of Ag 6 in one case, and of Ag 15 in six cases. In three out of 22 children we detected sensitivity to horse serum albumin. It appears that the most sensi-

tive patients (reaction to horse extract in histamine release) reacted to a larger number of allergenic molecules.

Fig. 1 shows the allergen dose response curves for histamine release from the leukocytes of two horse allergic individuals. Patient M.H. reacted

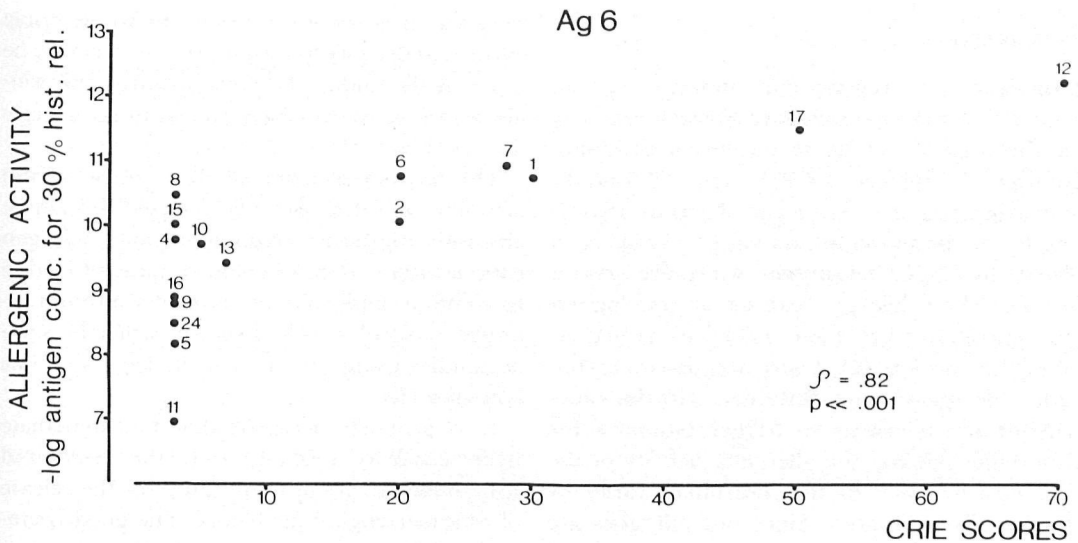


Fig. 4. Correlation between allergenic activity of horse Ag6 as determined by histamine release from washed leukocytes and CRIE using an arbitrary score system. Numbers indicate patient identity.

only to the four epidermal allergens and gave no histamine release on incubation with horse serum albumin. Ag 9 has the highest allergenic activity, since on a molar basis the lowest concentration (6×10^{-2} M) is needed for 30% histamine release. In contrast, patient G.W. was most sensitive to Ag 11, followed by Ags 6, 9, and 15. In this patient horse serum albumin has the capacity to induce histamine release, although it appears to be of relatively low allergenic activity compared with the four epidermal allergens.

In Fig. 2 the allergenic activities of the allergens, expressed as the molar concentration which was necessary to induce 30% histamine release are summarized in all 22 patients. The highest mean activity was found for Ag 11 (4×10^{-11} M), followed by Ag 9 (2×10^{-10} M), Ag 6 (5×10^{-10} M), Ag 15 (2×10^{-9} M) and horse serum albumin (6×10^{-7} M).

The heterogeneity of the individual patterns of sensitization (allergoprints) was confirmed by CRIE. Fig. 3 presents seven individual allergoprints plus the CRIE-data obtained from a control-serum pool of 1,000 normal blood donors. Patient No. 1 gave a precipitate with Ags 6, 11, and 15, patient No. 2 with Ags 6, 9, 11, and 15,

patient No. 4 with Ags 11 and 15 only, patient No. 12 with all the purified allergens plus others, and patient No. 16 with one antigen (Ag 11) only.

A possible correlation between the amount of serum-IgE-antibodies being bound by the various allergens in CRIE and the capacity of these allergens to induce histamine release from washed leukocytes was investigated. The CRIE-score for each antigen was plotted against the allergenic activity, as determined by histamine release. The data for Ag 6 are shown in Fig. 4. It is evident that patients who produce a 30% histamine release at very low allergen concentrations, tend to produce precipitates with their serum IgE-antibodies early on, and therefore have high CRIE scores. The correlation coefficient was $r = 0.82$ ($P < 0.001$) for Ag 6, $r = 0.74$ ($P < 0.001$) for Ag 9, $r = 0.47$ ($P < 0.05$) for Ag 11, and $r = 0.73$ ($P < 0.001$) for Ag 15. There are, however, a number of patients with insignificant CRIE score and a 30% histamine release to allergen concentrations between 10^{-7} and 10^{-11} M, most probably due to the content of minor allergenic impurities in the isolated allergenic preparations (Fig. 4).

DISCUSSION

Our histamine release data indicate that all four epidermal proteins have allergenic activity in the majority of horse sensitive individuals. Similarly by means of CRIE Ags 6, 9, and 11 had been found to be major allergens according to the definition (6). As has previously been shown by CRIE, the proteins which are present in the whole allergen extract at the highest concentration (Ags 9 and 11) have the highest allergenic activity (4). There are, however, considerable quantitative differences in the capacity of the allergens to trigger basophils for histamine release: the allergenic activity of the proteins in some of the individuals varies by two to three decades. Since our allergens are contaminated by other proteins from 0 to 5% (w/w) (4), a certain contribution to the histamine release, which is induced by an allergen concentration considerably higher than that of the most active allergen and with an insignificant CRIE score, must be due to impurities and therefore cannot be regarded as significant in all cases.

The activity patterns (allergoprints) are different from one patient to another. Information on the number of allergens in an extract, including their allergenic activity in patients, is a prerequisite for steps of extract purification and for standardization. Besides information about the total allergenic activity, content of important allergenic molecules must be added. Furthermore, for future hyposensitization treatment the variability of the individual allergoprints has to be taken into consideration, before the optimal treatment extract is chosen, supposing that patients' immune responses are specific as recently shown by Østerballe (13).

It appears that the most sensitive allergic individuals tend to react to a larger number of allergens than patients with a lower degree of sensitivity. Leukocytes from patients with a significant histamine release to only two or three epidermal allergens needed higher concentrations of the whole horse extract in order to produce 30% histamine release than those with a sensitivity to all four allergens. Two of our three children allergic to horse serum albumin

were the most sensitive ones to the whole horse extract. Sensitivity to serum proteins seems to be more likely among the most sensitive individuals, as has previously been shown in rat allergic donors (11).

The leukocytes from all three horse serum albumin sensitive patients released histamine also with dog serum albumin at similar allergen concentrations. This phenomenon might be due to common antigenic determinants on both albumin molecules, as was shown earlier by similar studies using the albumin molecules of rats and mice (12).

It is generally accepted that for immediate hypersensitivity reactions only the cell-bound IgE-antibodies are of significance for the release of pharmacological mediators. The good correlation of the semiquantitative CRIE as an assay system which determines IgE-antibodies to individual allergens in serum, and histamine release as a quantitative assay for detecting cell-bound IgE-antibodies, indicates that the amount of cell-bound antibodies is well reflected by the concentration of IgE-antibodies in the serum.

For testing the allergenic activity of purified proteins the histamine release assay obviously is more sensitive: The low or negative CRIE – scores of a large number of our patients, who show significant histamine release from leukocytes with allergen concentration between 10^{-9} and 10^{-7} M, are, however, explained as due to impurities in the isolated allergenic molecules. For determinations of patients' allergoprints to complex allergenic mixtures such as extracts, data which correlate statistically significantly with the more accurate histamine release data can be revealed without availability of purified fractions. A combination of CRIE and histamine release is well suited to control the purity of isolated fractions.

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