

Allergenic activity of cat and dog skin fractions obtained by Sephadex gel filtration

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Summary

Allergens derived from domestic mammals have been investigated by subjecting extracts of cat and dog skin to Sephadex gel filtration and assessing the activity of the eluted fractions by skin tests in appropriate allergic subjects. A certain heterogeneity of allergenic activity occurred and minor allergens, with an estimated molecular weight greater than 70,000, were found which showed activity in patients highly sensitive to cat and dog serum proteins. The major allergens of both mammalian skin extracts were eluted in similar positions between protein markers of 24,000 and 67,000 molecular weight.

Introduction

The dander from domestic animals is an important cause of asthma and rhinitis but, although detailed studies on horse dander extracts have been made by Squire (1950) and Stanworth (1957a, b, 1964, 1965), few attempts at characterization of the allergens from cat and dog dander have been made since the early work of Wodehouse (1917a, b).

The major recent study is that by Varga & Ceska (1972) who fractionated extracts of cat and dog dander by isoelectrofocusing in polyacrylamide gel. An investigation of the value of another technique, Sephadex gel filtration, in the study of cat and dog allergens was undertaken, as it has been reported to be a useful procedure in the fractionation of horse dander (Stanworth, 1965) and has also been widely used in studies of the allergens of ragweed and grass pollens. Cat and dog skin has been used for extraction in the present study, since skin was more easily obtained in the required quantity than dander, and yielded extracts which contained a high level of allergenic activity when compared with commercial dander extracts by cutaneous testing. In view of the presumed high serum protein content of the extracts, preparations of serum were also tested to determine whether additional allergens were present in the skin extracts.

In the work to be reported here, extracts of cat and dog skin were fractionated by

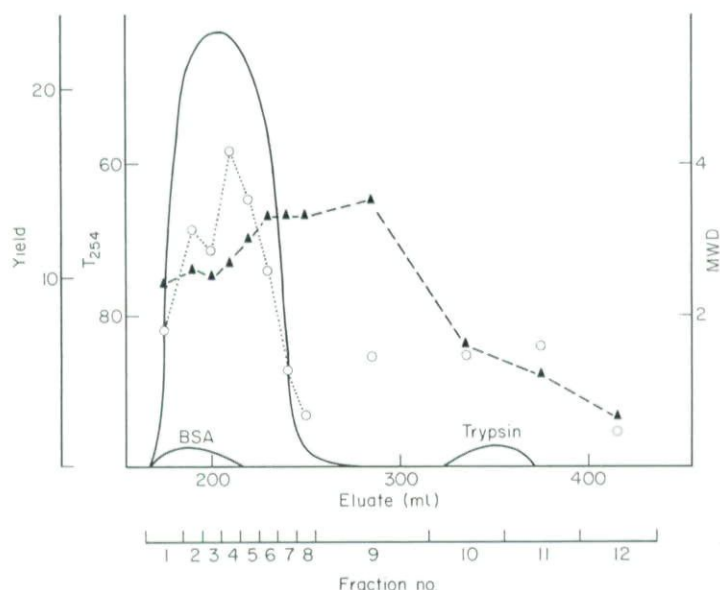


Fig. 1. Cat skin: gel filtration on Sephadex G-100. —, % transmission at 254 nm; ○, % of weight yield; ▲, MWD.

gel filtration on Sephadex G-100 and G-200 and the distribution of allergenic activity in the eluate was assessed by skin prick testing in allergic subjects.

Materials and methods

Extracts

Cat and dog skin, obtained from laboratory animals, was minced, defatted in ether and extracted in Coca's solution for 7 days. The liquid extract was Seitz filtered,

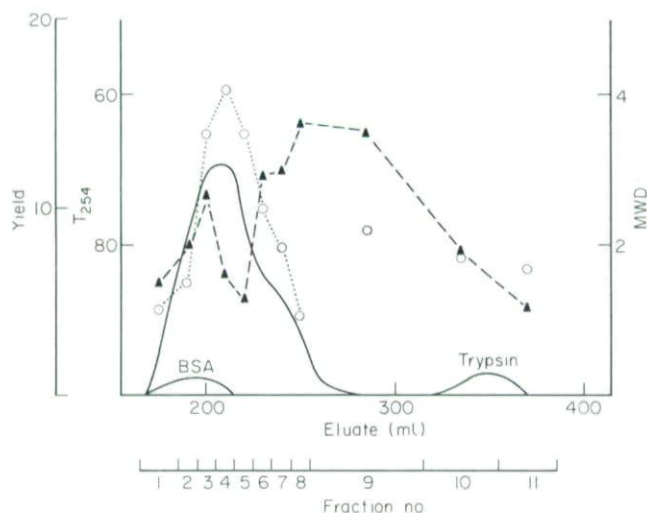


Fig. 2. Dog skin: gel filtration on Sephadex G-100. —, % transmission at 254 nm; ○, % of weight yield; ▲, MWD.

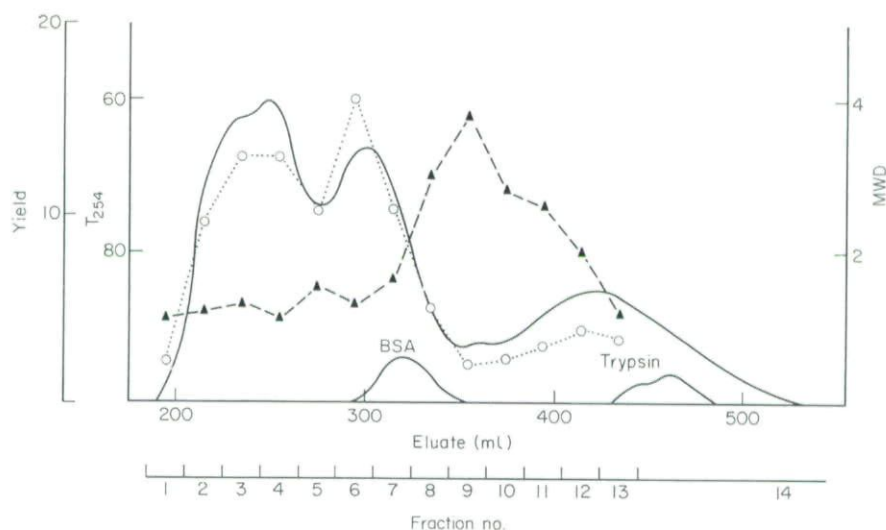


Fig 3. Cat Skin: gel filtration on Sephadex G-200. —, % transmission at 254 nm; ○, % of weight yield; ▲, MWD.

dialysed and freeze dried and the resulting material was reconstituted in 0.02 M ammonium bicarbonate, passed through Sephadex G-25 (Pharmacia, Uppsala) and freeze dried. Cat and dog serum was Seitz filtered, dialysed and freeze dried and passed through Sephadex G-25 as described above.

Gel filtration

(a) *Sephadex G-100*. 5 ml of a 20 mg/ml solution of the extract was applied to a column (3 × 90 cm) of Sephadex G-100 (Pharmacia, Uppsala) in 0.1 M ammonium bicarbonate. Elution was performed at 4°C using upward flow from a peristaltic pump, flow rate 14 ml/hr. The absorption of the eluate at 254 nm and 280 nm was recorded continuously (Uvicord flow analyser, LKB), yielding essentially similar curves at each wavelength, and fractions of 10 ml were collected, bulked together as indicated (Figs. 1 and 2) and freeze dried.

Bovine serum albumin and trypsin were separately passed through the column in the same way and their elution positions were determined by absorption at 280 nm and by proteolytic enzyme activity of the eluate respectively.

(b) *Sephadex G-200*. Gel filtration was performed in a similar way to that described above for G-100, but using a flow rate of 13 ml/hr and applying 5 ml of a 40 mg/ml solution of each extract (Figs. 3 and 4).

Skin testing

Solutions of the freeze dried extracts were prepared at 5 mg/ml in Coca's solution containing 50% v/v glycerol, heated for 30 min at 56°C to inactivate viruses, and filtered through 0.45 µm Millipore membranes.

Subjects with asthma and/or rhinitis with a history of sensitivity to cat or dog and/or a positive skin test to a commercial cat or dog extract were tested by the prick method, the weal diameter being measured after 15–20 min. Subjects who gave a reaction to at least one of the test solutions were included in the tables and the mean

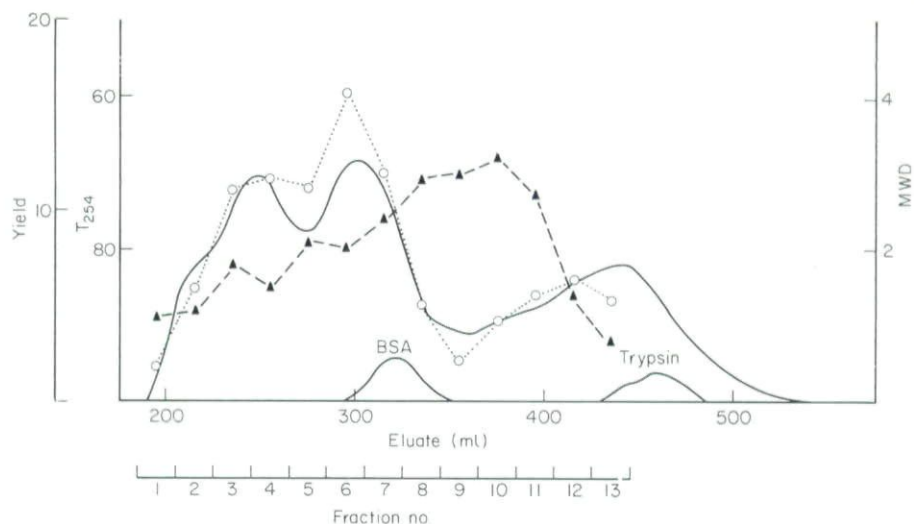


Fig 4. Dog skin: gel filtration on Sephadex G-200, —, % transmission at 254 nm; ○, % of weight yield; ▲, MWD.

weal diameter (MWD) obtained for each extract was calculated, this value being also expressed as a percentage of the mean weal diameter obtained for the unfractionated material. The solutions elicited no skin reactions in control subjects.

The variability of the prick test was assessed by carrying out tests in each of twenty-one subjects with sixteen aliquots of one allergen solution. The mean weal diameter elicited by each aliquot was calculated and the mean of these values was found to be 2.0 mm, SD 0.23. Thus the coefficient of variation $[(SD \times 100)/\text{mean}]$ for the MWD values was 11.4%, an acceptably low level.

Analytical procedures

Nitrogen estimation was carried out by the micro-Kjeldahl method and hexose estimation by the orcinol method expressing the hexose content as galactose (Kabat & Mayer, 1961).

Results

(a) Sephadex G-100 fractionation

The fractions from gel filtration of cat skin extract on Sephadex G-100 were tested in comparison with unfractionated cat skin and serum preparations (Table 1, Fig. 1), the cat skin extract eliciting larger weals and reacting in a larger number of subjects than did the cat serum. A fairly high level of allergenic activity was found in all the cat skin fractions 1–10, with a peak of activity in fractions 6–9, which decreased to a value below that for cat serum in subsequent fractions. The absorption curve at 254 nm showed a single peak of absorption after the passage of the void volume, which coincided with the maximum yield, but preceded the peak of allergenic activity. The total nitrogen content of the fractions showed a gradual fall with no apparent correlation with the allergenic activity, the allergenically most active fractions 6–9 having nitrogen contents of 10.0–13.2% and hexose contents ranging from 2.5 to 10.5%.

Table 1. Cat skin: gel filtration on Sephadex G-100. Skin tests in eighteen subjects

Extract (5 mg/ml)	% of yield	Hexose (%)	N (%)	No. positive	% positive	MWD	MWD for fr. $\times 100$
							MWD for*
Skin		3.3	16.0	16	89	3.0	*
Fraction 1	7.14	2.9	16.4	12	67	2.4	80.0
Fraction 2	12.5	2.3	15.9	12	67	2.6	86.7
Fraction 3	11.4	2.3	15.4	12	67	2.5	83.3
Fraction 4	16.7	2.2	14.5	13	72	2.7	90.0
Fraction 5	14.1	2.0	14.1	14	78	3.0	100
Fraction 6	10.3	2.5	13.2	15	83	3.3	110
Fraction 7	5.10	3.3	12.3	14	78	3.3	110
Fraction 8	2.64		12.8	17	94	3.3	110
Fraction 9	5.85		10.0	16	89	3.5	117
Fraction 10	5.86	NA	9.20	12	67	1.6	53.3
Fraction 11	6.43	NA	4.43	11	61	1.2	40.0
Fraction 12	1.97	NA	2.03	7	39	0.63	21.0
Serum		2.9	NA	10	56	2.1	70.0

Hexose and N estimations performed on fractions obtained from another run. NA, Not available.

Gel filtration of dog skin extract (Table 2, Fig. 2) produced two peaks of allergenic activity, one (fractions 2-4) coinciding with the ascending slope of the main u.v. absorption peak and one (fractions 6-10) in a similar position to that found on gel filtration of cat skin. The most active material from both cat and dog skin extracts was eluted in a position between that of the molecular weight markers bovine serum albumin (mol. wt 67,000) and trypsin (mol. wt 23,800).

(b) *Sephadex G-200 fractionation*

The fractions from G-200 gel filtration of cat skin extract were tested in twenty-two subjects (Table 3, Fig. 3). As with G-100 fractionation, a fairly high level of activity

Table 2. Dog skin: gel filtration on Sephadex G-100. Skin tests in ten subjects

Extract (5 mg/ml)	% of yield	No. positive	% positive	MWD	MWD for fraction $\times 100$
					MWD for*
Skin		9	90	2.2	*
Fraction 1	4.25	6	60	1.5	68.2
Fraction 2	8.52	7	70	2.0	90.9
Fraction 3	13.8	7	70	2.7	123
Fraction 4	16.2	7	70	1.6	72.7
Fraction 5	12.8	6	60	1.3	59.1
Fraction 6	9.93	6	60	2.9	132
Fraction 7	7.48	8	80	3.0	136
Fraction 8	4.17	9	90	3.6	164
Fraction 9	8.91	10	100	3.5	159
Fraction 10	7.32	8	80	1.9	86.4
Fraction 11	6.68	6	60	1.2	54.5
Serum		5	50	1.4	63.6

Table 3. Cat skin: gel filtration on Sephadex G-200. Skin tests in twenty-two subjects

Extract (5 mg/ml)	% of yield	Hexose* (%)	N (%)	No. positive	% positive	MWD†	MWD for fr. × 100
							MWD for*
Skin		3.3	16.0	15	68	1.5	*
Fraction 1	2.1	3.3	18.8	9	41	1.1	73.3
Fraction 2	9.5	2.7	16.3	10	46	1.2	80.0
Fraction 3	13	2.2	14.4	9	41	1.3	86.7
Fraction 4	13	2.2	14.3	9	41	1.1	73.3
Fraction 5	11	2.3	15.6	11	50	1.5	100
Fraction 6	16	1.8	15.9	9	41	1.3	87.0
Fraction 7	11	2.5	11.8	13	59	1.6	107
Fraction 8	4.9	4.9		18	82	3.0	200
Fraction 9	2.0	8.4		19	86	3.8	253
Fraction 10	2.3		20	90	2.8	187	
Fraction 11	3.0		14	64	2.6	173	
Fraction 12	3.9	7.8	10.0	11	50	2.0	133
Fraction 13	3.5			10	46	1.2	80.0
Fraction 14	3.5	NA	NA	9	41	0.72	48.0
Serum		2.9		5	23	1.0	66.7

NA, Not available.

* Hexose estimations carried out on fractions obtained from another run.

† The difference between the MWD for fractions 1 and 9 is significant at the 5% level (two way analysis of variance using Tukey's method of comparing the group means).

was found in all fractions, the mean diameters of the weals elicited by each of G-200 fractions 1-13 being greater than 70% of that produced by the unfractionated extract. Fractions 8-12 showed the highest activity, the value of mean weal diameter for fraction 9 being more than twice that for the original extract and more than three times that for fraction 1. Individual hexose and nitrogen estimations were not carried out on fractions 7-14, but the values for fractions 7-12 ranged from 2.5 to 7.8% hexose and for fractions 7-14 from 10.0 to 11.8% nitrogen.

The dog skin fractions showed a similar pattern of activity (Table 4, Fig. 4) with most allergenic activity in fractions 7-11, fraction 10 being most active.

For convenience, fractions 1-6 from G-200 gel filtration of cat and dog skin extracts have been designated 'group 1 allergens' and the more active fractions 7-12 'group 2 allergens'. These more active fractions from cat and dog skin were again eluted in a position between that of trypsin and bovine serum albumin, indicating a molecular weight between 23,000 and 67,000. This is, of course, only a very approximate estimation as other properties of the molecules other than molecular weight can selectively affect their degree of retardation on Sephadex. There was very little material which was eluted later than trypsin and it was difficult to obtain sufficient quantity for comparative skin testing. However, the last fraction from cat skin (fraction 14) showed low allergenic activity.

In the group of patients tested, some variation in the pattern of response was found, suggesting the presence of multiple allergens. The individual responses of two patients to cat skin fractions (G-200) are shown in Fig. 5. Although subject A gave larger skin weals to fractions 7-9 than did subject B, he did not respond to fractions

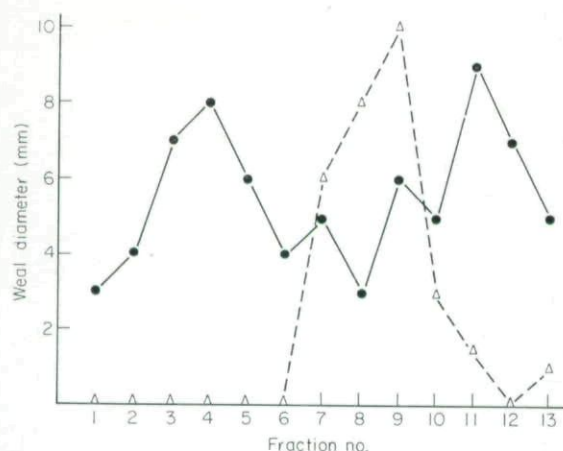


Fig. 5. Cat skin: individual responses of two subjects (A, Δ ; B, $\bullet$) to G-200 fractions.

1-6, whereas subject B reacted fairly strongly to these earlier fractions. With one exception all the twenty-two patients reacted to at least two fractions in the region 7-13 ('group 2 allergens'), but there seemed to be an association between a reaction to cat serum and a strong reaction extending to the earlier high molecular weight fractions ('group 1 allergens'). When five subjects reacting to cat serum were excluded from Table 4, the higher activity of the 'group 2 allergens' was more marked (Table 5).

Five patients with a clinical history of sensitivity to cat and dog were tested with fractions from G-200 separation of both cat and dog skin, and showed a similar pattern of reactivity to both series of fractions; two subjects reacted only to the 'group 1 allergens' and three reacted to all fractions and to both cat and dog serum.

Table 4. Dog skin: gel filtration on Sephadex G-200. Skin tests in fifteen subjects

Extract (5 mg/ml)	% of yield	No. positive	% positive	MWD	MWD for fraction $\times 100$
					MWD for*
Skin		8	53	1.9	*
Fraction 1	1.83	8	53	1.1	57.9
Fraction 2	5.87	7	47	1.2	63.2
Fraction 3	11.1	6	40	1.8	94.7
Fraction 4	11.7	6	40	1.5	79.0
Fraction 5	11.2	8	53	2.1	111
Fraction 6	16.2	6	40	2.0	105
Fraction 7	12.0	7	47	2.4	126
Fraction 8	5.13	11	73	2.9	153
Fraction 9	3.13	12	80	3.0	158
Fraction 10	4.24	12	80	3.2	168
Fraction 11	5.63	13	87	2.7	142
Fraction 12	6.42	9	60	1.4	73.7
Fraction 13	5.33	5	33	0.80	42.1
Serum		5	33	1.8	94.7

Table 5. Cat skin: gel filtration on Sephadex G-200. Skin tests in seventeen subjects; excluding reactors to cat serum

Extract (5 mg/ml)	No. positive	% positive	MWD	MWD for fraction $\times 100$
				MWD for*
Skin	10	59	0.88	*
Fraction 1	5	29	0.32	36.4
Fraction 2	6	35	0.47	53.4
Fraction 3	6	35	0.44	50.0
Fraction 4	4	24	0.26	30.0
Fraction 5	7	41	0.65	73.9
Fraction 6	5	29	0.44	50.0
Fraction 7	9	53	1.2	136
Fraction 8	13	77	2.4	273
Fraction 9	14	82	3.2	364
Fraction 10	15	88	2.3	261
Fraction 11	9	53	1.5	170
Fraction 12	6	35	0.82	93.2
Fraction 13	5	29	0.50	56.8
Fraction 14	5	29	0.40	45.5

These different patterns of reactivity did not seem to be associated with the degree of clinical sensitivity of the patient to either cat or dog, other sensitivities, the age of onset of symptoms or with hyposensitization treatment.

Discussion

Gel filtration has been of value in the fractionation of horse dander, Stanworth having reported that the major horse dander allergen prepared by salt precipitation and starch gel electrophoresis could be separated from an inactive contaminant by the use of Sephadex G-75 (Stanworth, 1957a, 1965). The present study of cat and dog allergens used skin rather than dander extracts and it was therefore necessary to test serum, in order to check that the reactions obtained to the skin extracts were not solely due to their serum protein content. In general, the skin extracts were found to be more reactive than the serum preparations, although the group of patients tested to the dog skin G-200 fractions proved to be exceptional in their high reactivity to dog serum; this proportionately high reactivity to serum has not been found in most patients tested in further studies.

Fractionation of cat and dog skin on Sephadex G-100 indicated that some activity was present in the high molecular weight fractions, in addition to that obtained in the main peak of allergenic activity. Sephadex G-200 was therefore used to investigate this further and was useful in separating an allergenically active fraction of cat and dog skin which contained constituents of estimated molecular weight between 23,000 and 65,000, probably in the range 30,000–40,000. However, although the proportional activity was greatest in this fraction, activity also occurred in material of a higher molecular weight. The variation in the pattern of response by individual subjects suggests that components with different allergenic specificities may be important in some allergic subjects. A slight association between a strong reaction to the serum preparation and a reaction, including the higher molecular weight 'group 1 allergens'

as well as the more active 'group 2 allergens' of G-200 fractionation, was noted. It seems probable that the 'group 2 allergens' described here for cat and dog skin correspond to the active allergenic material which Stanworth obtained from horse dander and reported as having a molecular weight of approximately 34,000 (Stanworth, 1965). The minor 'group 1 allergens' may be present in dander in only small quantities, although sufficient for immunological reactivity, or the larger number of patients available for testing here may have increased the probability of including subjects with reactions to minor allergens.

Varga & Ceska (1972) fractionated cat and dog dander extracts by isoelectrofocusing and assessed the activity of the fractions by the radio-allergosorbent test (RAST) using sera from four patients. They found that the major allergens of cat dander were in the narrow pI range 4.5–5.0 and of dog dander pI 4.3–4.7, but that minor allergenic components with pI values ranging from 3.0–8.5 reacted with certain sera. A similar variation in activity has been found for fractions obtained by DEAE-cellulose chromatography of cat skin extract (Holford-Strevens, 1972). The most active fraction migrated with an electrophoretic mobility similar to that of serum albumin, but a fraction including a component with electrophoretic characteristics of serum gamma globulin was active in three of the fourteen patients tested.

Using gel filtration on Sephadex in combination with other procedures, extracts of pollens have also been shown to contain more than one group of allergens. Johnson & Marsh (1965) have shown that rye grass pollen contains two groups of allergenic components with molecular weights of approximately 30,000 and 10,000 respectively, which have different allergenic specificities as judged by skin testing and by their behaviour in releasing histamine *in vitro* from leucocytes of allergic subjects (Lichtenstein, Marsh & Campbell, 1969). Kisil, Gyenes & Schon (1971) have also found heterogeneity of skin active components of ragweed pollen, demonstrating by P-K neutralization techniques at least five allergic components in fractions obtained by electrophoretic separation of the extract.

It appeared that the mammalian skin extracts were unusual in containing allergenic components with a molecular weight above 70,000, although these represented only minor allergens, as the allergenic activity of most inhalant materials has been reported as below this value. The corresponding high molecular weight fractions of the mite *Dermatophagoides farinae*, for example, showed only low activity in house dust sensitive patients whereas molecules of probable weight 20,000–30,000 represented the major allergens (Holford-Strevens, 1972; Belin & Holford-Strevens, unpublished). Although the cat and dog skin extracts showed some heterogeneity the major allergens from both animals showed an elution profile indicating that their molecular weight lay in the region 30,000–40,000 mol. wt, which is consistent with the findings of many authors that the major inhalant allergens lie in a somewhat restricted molecular weight range. Further work is in progress to investigate the relationships between the 'group 1' and 'group 2' allergens of the skin extracts.

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