

Identification of Major Allergens from the House Dust Mites, *Dermatophagoides Farinae* and *Dermatophagoides Pteronyssinus*, by Electrophoretic Blotting

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The allergens were separated from the extracts of house dust mites by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and identified by autoradiography. Over 30 protein bands of the whole body extract of *Dermatophagoides farinae* were apparent on 10-20% gradient SDS-PAGE, and 13 bands with MW between 93KD and 12KD bound with specific IgE antibodies in patients' sera sensitive to house dust mites. The major allergenic component of the whole body extract of *D. farinae* was the protein of MW 14-15KD, which was detected in 95.7% of 47 patients' sera sensitive to house dust mites. The extract of *Dermatophagoides pteronyssinus* supplied by Bencard Company, England was thought to contain feces enriched material as noted in a few broad protein bands on SDS-PAGE. Seven allergenic components were shown by autoradiography. The protein band of MW 14-15KD was one of the most frequently revealed allergens on autoradiography, which has appeared in 32.5% of 40 patients' sera sensitive to house dust mites. The electroblotting technique used in the present study was fast, convenient and highly useful for both the identification of allergen components and the screening of specific IgE antibody. The individual variations of IgE immune responses to the allergenic components of the two house dust mites were discussed.

Key Words: House dust mites, major allergen, electroblotting

House dust mites, especially *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, have been shown to be the most important allergens in hypersensitivity to house dust by Voorhost *et al.* (1964). Since then, a lot of research on clinical, epidemiologic, taxonomic, biologic and immunologic aspects of house dust mites has been done in many countries (Maunsell *et al.* 1968; Spieksma 1970; Miyamoto *et al.* 1970; Murray and Zuk 1979; Vervloet *et al.* 1982; Platts-Mills *et al.* 1987).

House dust mites, *Dermatophagoides* species, have been found in house dust in this country since

1977 (Cho and Houh 1987). Two species of *Dermatophagoides* inhabit house dust together but *D. farinae* is more prevalent. House dust mites are the most prevalent aeroallergens reactive in Korean respiratory allergic patients (Kang 1973; Whang *et al.* 1974; Yoon *et al.* 1989).

Most patients are reactive to both kinds of house dust mites, but *D. farinae* causes somewhat stronger and more prevalent skin reactions than *D. pteronyssinus* does (Yoon *et al.* 1989).

In the house dust mites about 30-50 antigens and 20-30 allergens were noted by CRIEST and immunoblot method (Le Mao *et al.* 1983; Arlian *et al.* 1987; Baldo *et al.* 1989). Among allergenic components of house dust mites extracts, IgE binding components varied with atopic individuals (Krilis *et al.* 1984; Baldo *et al.* 1989).

Therefore, we attempted to study the important allergenic components of house dust mites in the patients allergic to house dust mites in this country.

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MATERIALS AND METHODS

Serum specimens

Sera were obtained from respiratory allergy patients who showed strong skin reactivity to the allergens of house dust mites and class 4 of the radioallergosorbent test (RAST) to both house dust mites, and who visited the Department of Internal Medicine in Severance Hospital of Yonsei University College of Medicine. The sera were stored at -20°C until used. None of the patients were receiving allergen immunotherapy. Control sera were obtained from patients who showed negative response to 50 inhalant allergens on the skin prick test.

Extracts of house dust mites

Dermatophagoides farinae was purely cultured in media mixed with mouse foods and dried yeast in our laboratory. The bodies of *D. farinae* were harvested by sieving. The clean mite bodies were collected on defatting with ethylether overnight at 4°C and dried.

Some of the dried raw materials of *D. farinae* and *D. pteronyssinus* were supplied from Bencard Company, England. They were fine granule-like substances with rare mite bodies and seemed to contain a lot of mite feces. The Bencard materials were also defatted with ethylether before extraction. The extraction methods were modified as described by Nakada *et al.* (1985). One gram of the source materials was extracted and stirred continuously with 100 ml of phosphate buffered saline (PBS) for 48 hours at 4°C .

Then the extract was centrifuged at 10,000g for one hour at 4°C and the supernatant was dialyzed in distilled water for 48 hours. The contents were lyophilized and stored in a refrigerator until used.

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis

This was carried out in 16 cm long 10-20% gradient acrylamide vertical slab gels using the discontinuous buffered system of Laemmli (1970). Mite extracts (50 mg/ml, 1.0 ml), dissolved in sample buffer without mercaptoethanol [500 μl of 1M Tris (pH 6.8), 1,600 μl of 50% glycerol, 640 μl of 25% SDS, 40 μl of bromophenol blue (100 mg/ml) and 820 μl of distilled water] and boiled for 90 seconds in boiling water bath, were laid over the stacking gel

of 4% SDS-polyacrylamide gel. The stacking gel had a single slot at one end which was used for molecular weight markers. Electrophoresis was carried out at 30mA of constant current and stopped when the marker dye reached the bottom of the gel. After electrophoresis, a strip including the molecular weight marker was cut from the gel, stained with Coomassie brilliant blue R-250 (CBB; Bio-Rad Laboratories) and destained with destaining solution (600 ml of distilled water, 300 ml of methanol and 100 ml of glacial acetic acid).

Electrophoretic transfer of protein on nitrocellulose membrane

The protein bands of SDS-polyacrylamide gel after electrophoresis were transferred to nitrocellulose membrane (0.45 μ pore TM-NC4 Roll, Hoeffer Scientific Instruments, San Francisco) using a modified method of Towbin *et al.* (1979) as described by Sutton *et al.* (1982).

Transfer was done at room temperature with a constant voltage of 50V for 16 hours using a transfer electrophoresis unit with power lid (Hoeffer Scientific Instruments, San Francisco). The transfer buffer was 6.25 mM Tris-1.4% glycine in 20% methanol. After transfer, a strip of nitrocellulose was cut and stained with CBB. The membrane was washed for one hour in 10% methanol in PBS with rocking to remove the SDS, followed by a 15 minute wash in PBS and then rocking for two hours in 0.1% Tween 20 in PBS (TPBS) to block unbound membrane sites (Bateiger *et al.* 1982). The nitrocellulose membrane was dried in the cold air and stored in this condition.

Probing the nitrocellulose membranes with patients' sera

The procedures were modified as previously described (Ford and Baldo 1987; Park *et al.* 1989). Membranes were cut into 5 mm wide strips, numbered with a ballpoint pen, and placed in flat plastic boxes. The membrane strips which transferred the proteins of the extracts of *D. farinae* (Yonsei material) were incubated with 47 patients' sera diluted as 1 to 4 with 10% new born calf serum for 18 hours at room temperature. And the membrane strips which bound the transferred proteins of the extract of *D. pteronyssinus* (Bencard material) were incubated with 40 sera samples and two negative control sera diluted as 1 to 4 with 10% newborn calf serum for 18 hours at room temperature.

Twenty-two sera were tested with two kinds of

membrane strips, one with *D. farinae* allergen and the other with *D. pteronyssinus*. Ten sera were incubated with two strips with *D. farinae* allergens, for evaluation of the effects of different incubation time on autoradiography.

The membrane strips treated with sera were washed with TPBS and incubated with anti-IgE RAST tracer (300,000 cpm per strip, Phadebas, Sweden) for 18 hours at room temperature. Then the strips were washed with TPBS and dried in room air.

Autoradiography

The membrane strips treated through all steps were exposed to X-ray film (Fuji Company, Japan) in X-Omatic cassette at (-70°C) for 7 days. Ten strips treated with 10 different sera were exposed in a different cassette for 7 and 14 days simultaneously. The films were developed and the bands were examined carefully by the naked eye.

RESULTS

SDS-PAGE of the extracts of house dust mites

Fig. 1 shows the protein bands of SDS-PAGE of house dust mite extracts. The extract of whole bodies of *D. farinae* (Yonsei materials) shows numerous protein bands (over 30) (Fig. 1. lane B). Protein bands of *D. farinae* corresponding to the MW of 45KD or below were not very well separated on SDS-PAGE. The extract of *D. farinae* (Bencard material, Lane C) shows about 15 protein bands having MW of 29 KD or above with three diffuse wide bands below that. The extract of *D. pteronyssinus* (Bencard material, Lane D) shows a few bands having MW of 45KD or above and 4 wide bands below 45KD.

IgE-binding components of *D. farinae* extract by autoradiography

Fig. 2 shows IgE-binding components of *D. farinae* extract from Yonsei material by autoradiography. Fig. 3 is a schematic drawing chart of IgE-binding bands of *D. farinae* (Yonsei) extract with 47 sera sensitive to house dusts mites. According to the density of autoradiography film, three different bands were drawn. The broad lines are the protein bands showing the strongest densities on autoradiography by the naked eye. On the autoradiography of *D. farinae* extract (Yonsei)-SDS-PAGE, there were 13 protein bands between 93KD and 12KD

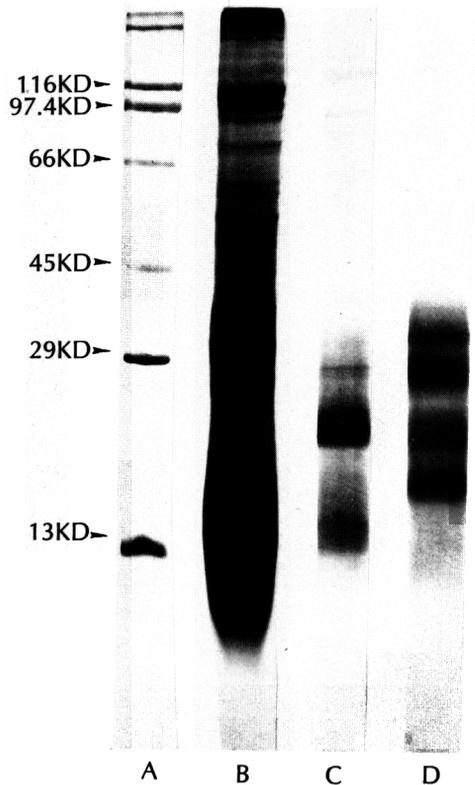


Fig. 1. The pattern of SDS-polyacrylamide gel electrophoresis using 10 to 20% gradient gel of the extracts of *D. farinae* (Yonsei Material, Lane B), *D. farinae* (Bencard Material, Lane C), *D. pteronyssinus* (Bencard Material, Lane D) and the marker proteins (Lane A).

which bound to IgE of patients' sera. There were no protein bands on autoradiography of the nitrocellulose strips which were reacted with three control sera. The protein band No. 12 (MW 14-15KD) was the most frequent IgE-binding band. Band No. 12 bound IgE in 95.7% of patients, No. 1 (MW 92-93KD) in 40.4%, No. 3 (MW 69-70KD) in 36.2% and No. 9 (MW 23-24KD) in 27.7% in order (Table 1). Band No. 12 showed the strongest signal in density for most sera except for two sera which were unbound to it. Band No. 9 (MW 23-24KD) demonstrated a moderately strong signal in density in the IgE-binding of 6 out of 13 sera. Band No. 13 (MW 12-13KD) showed IgE binding activity in one case (2.1%). Serum of case No 5 noted IgE binding in three protein bands with MW 44KD or above without any binding activity with band No. 12. One out

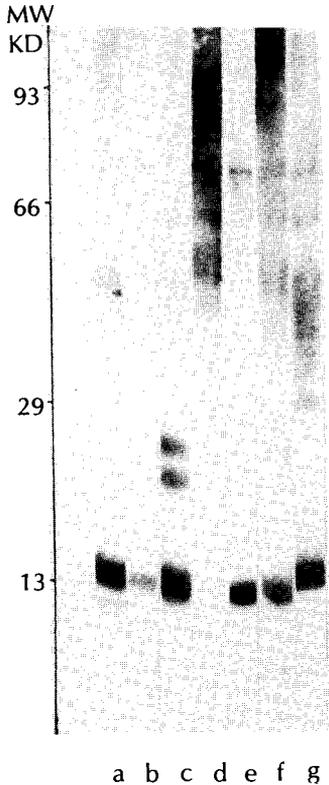


Fig. 2. Identification of the allergenic components of *D. farinae* bound with patients' sera (a-g) by autoradiography.

of 47 sera tested had no IgE-binding activity with protein bands of *D. farinae* extracts. Eleven sera out of 47 (23.4%) showed one band signal corresponding to the protein band No. 12 (MW 14-15KD) on autoradiography, indicative of their IgE-binding activity. The serum of case No. 6 was strongly reactive to *D. farinae* extracts and showed 8 protein bands on autoradiography including 4 bands with moderate to strong density. Most sera showed one to 5 protein bands on autoradiography (Table 2, Fig. 3).

IgE-binding components of *D. pteronyssinus* extract by autoradiography

Fig. 4. shows IgE-binding protein components of *D. pteronyssinus* extract from Bencard material. Seven protein bands bound with IgE in patients' sera. A band of MW 14-15KD was shown in 13 sera out of 40(32.5%) on autoradiography and it was the most prevalent one (Table 1). Seventeen sera out of 40(42.5%) did not show any IgE-binding activity on autoradiography. Sixteen(40%) sera showed one band bound with their IgE, which was a band of MW 14-15KD(Table 1 & 2). Each of the bands of MW 28-29KD and MW 23-24KD was shown in 9 sera(22.5%). One serum demonstrated 4 bands bound with its IgE. Two control sera showed no visible bands on autoradiography.

Comparison of IgE-bound components between *D. farinae* and *D. pteronyssinus* to the same sera

Twenty-two sera were incubated with the nitro-

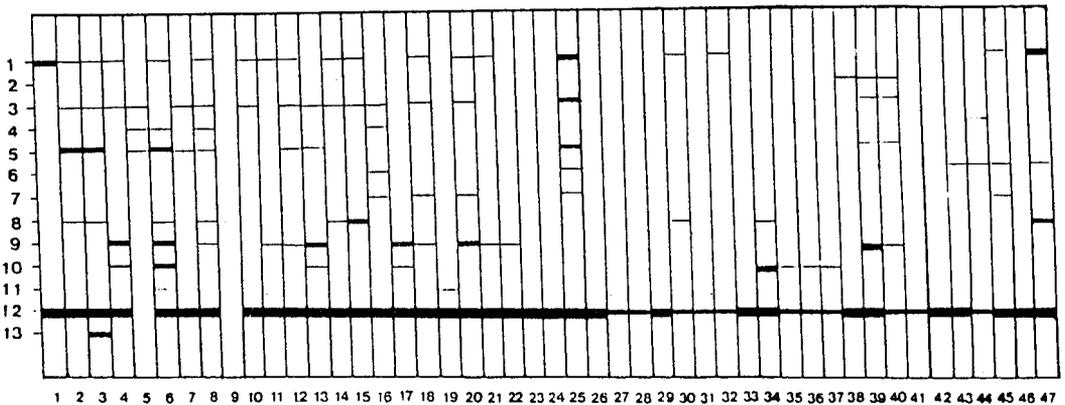


Fig. 3. A schematic chart drawn with the results of autoradiography of the nitrocellulose strips with the allergenic components of *D. farinae* treated with 47 patients' sera.

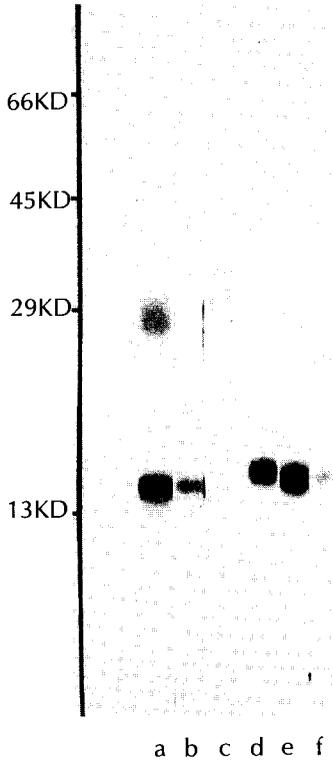


Fig. 4. Identification of the allergenic components of *D. pteronyssinus* bound with patients' sera (a-f) by autoradiography

Table 1. Allergenic component of the extracts of *D. farinae* and *D. pteronyssinus* bound with patients' IgE by immunoblot

Protein band No.	(Molecular weight)	No. of sera reacted (%)	
		<i>D. farinae</i> (n=47)	<i>D. pteronyssinus</i> (n=40)
1	(92-93KD)	19 (40.4)	1 (2.5)
2	(78-80KD)	3 (6.4)	0
3	(69-70KD)	17 (36.2)	0
4	(54-55KD)	5 (10.6)	0
5	(44-45KD)	11 (23.4)	0
6	(37-38KD)	6 (12.8)	1 (2.5)
7	(31-32KD)	5 (10.6)	2 (5.0)
8	(28-29KD)	9 (19.1)	9 (22.5)
9	(23-24KD)	13 (27.7)	9 (22.5)
10	(20-21KD)	8 (17.0)	0
11	(16-17KD)	2 (4.3)	0
12	(14-15KD)	45 (95.7)	13 (32.5)
13	(12-13KD)	1 (2.1)	0

cellulose strips bound with *D. farinae* allergens (Yonsei material) and *D. pteronyssinus* allergens (Bencard material) simultaneously. The quite different patterns of SDS-PAGE of the two house dust mites extracts (Fig. 1) made it difficult to interpret the results of autoradiographies performed with the same sera. Two protein bands (MW 28-29KD and MW 23-24KD) were demonstrated in a similar percentage of sera on autoradiography of both house dust mites. Nine sera out of 22 showed one or two bands in the extracts of both or either house dust mites bound with their IgE. Out of nine sera which had reacted with a protein band of MW 28-29KD, one serum showed equal positive reaction to both house dust mites and eight sera showed different patterns of autoradiography. Out of nine sera which had been reactive with the band of MW 23-24KD, one serum showed equal positive reaction to both house dust mites and eight sera showed different patterns of autoradiography (Table 3). According to the combinational analysis of reactive protein bands in two allergenic components of two house dust-mites, ten different patterns were noted (Fig. 5). It seems that IgE responses to these two antigenic components of two house dust-mites are variable in each patient.

The effect of exposure duration of autoradiography

Each of ten sera was incubated with two *D. farinae* transferred nitrocellulose membrane strips.

One part of the strips was exposed to X-ray film for 7 days and the other for 14 days. In general the bands of 14 days exposure resulted in stronger

Table 2. Number of allergenic components of *D. farinae* and *D. pteronyssinus* bound with patients' sera on autoradiography

Number of Allergenic components Bound	No. of sera (%)	
	<i>D. farinae</i> (%)	<i>D. pteronyssinus</i> (%)
0	1 (2.1)	17 (42.5)
1	11 (23.4)	16 (40.0)
2	8 (17.0)	3 (7.5)
3	9 (19.1)	3 (7.5)
4	5 (10.6)	1 (2.5)
5	9 (19.1)	0
6	2 (4.3)	0
7	1 (2.1)	0
8	1 (2.1)	0
Total	47 (100.0)	40 (100.0)

Table 3. Comparison of IgE-binding patterns to two allergenic components of *D. farinae* and *D. pteronyssinus* extracts in 22 sera

Allergenic Component (MW) **	Equal pattern		Non-equal pattern		Total
	D fa + *	D fa -	D fa +	D fa -	
	D pt +	D pt -	D pt -	D pt +	
28-29 KD	1	13	2	6	22
23-24 KD	1	13	3	5	22
Total	2	26	5	11	44

* D fa : *D. farinae*, D pt : *D. pteronyssinus*

** MW : Molecular weight

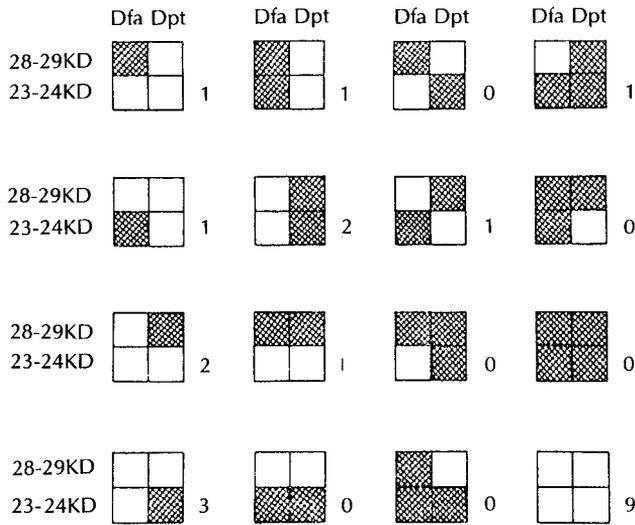


Fig. 5. The variety of IgE binding patterns of 22 sera to the two allergenic components (28-29KD and 23-24KD) of *D. farinae* (*D. fa*) and *D. pteronyssinus* (*D. pt*) by immunoblot.

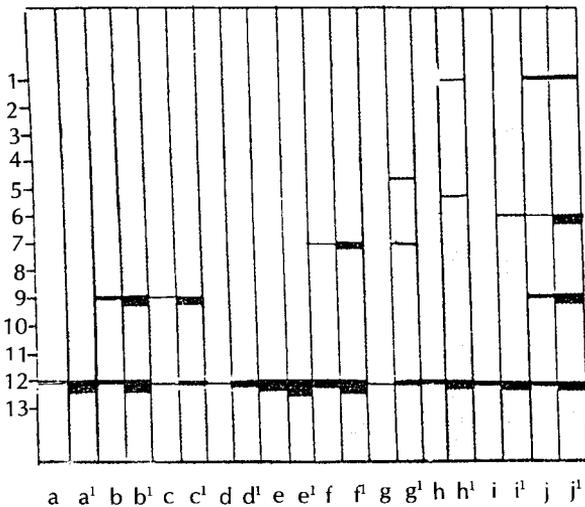


Fig. 6. The effect of exposure duration for autoradiography. One part of the strips (a-j) was incubated for 7 days and the other (a¹-j¹) for 14 days.

densities than those of 7 days exposure. In three of 10 sera new bands appeared after 14 days exposure. One band was newly developed in one serum (i & i') and two bands in two sera (g & g', h & h') in the 14 days of exposure (Fig. 6).

DISCUSSION

In 1967, Wide et al. developed a method to determine allergen specific IgE from patients' sera. For this crude or semipurified method, allergens have to be bound with beads, or activated paper disc or polystyrene tube, or nitrocellulose paper as a solid phase. For the determination of allergenic components from crude antigens, several techniques for separation of antigens have been developed. Crossed radioimmuno-electrophoresis (CRIE) showed extraordinary resolving powers in the analysis of allergenic components from crude antigens.

Krilis et al. (1984) showed 51 antigenic arcs on CIE of *D. pteronyssinus* and 11 allergenic arcs on CRIE. According to the criteria for major allergens defined by Löwenstein (1978) as "antigens that bind IgE from at least 50% of the sera of patients tested and demonstrated strong binding to at least 50% of these", they defined two allergens as major allergens. Alian et al. (1987) showed 35 antigens of *D. farinae* bodies, 33 antigens of *D. pteronyssinus* bodies and 20 antigens of *D. farinae* feces on CIE. And they showed 14 allergens in *D. farinae* bodies, 5 allergens in *D. pteronyssinus* bodies and 7 allergens in *D. farinae* feces on CRIE. In spite of these results CIE and CRIE have some critical disadvantages, such as good antiserum for each allergen, failure to detect allergens that are not precipitated by the antiserum and the problem of coprecipitation with precipitates situated beneath the specific IgE-binding precipitates (Krilis et al. 1984).

For allergen identification, another simpler method, the electrophoretic transfer or "western blotting technique", was introduced and applied (Towbin et al. 1979; Burnette 1981; Baldo 1982; Sutton et al. 1982).

Because of its sensitivity, resolving power, ease of use, high capacity and need for the employment of small quantities of extract, protein blotting is the method used frequently for identifying IgE-binding components in complex allergen extracts. With this system several allergen extracts, such as *D. pteronyssinus* (Sutton et al. 1982; Baldo 1982; Tovey and Baldo 1987; Tang et al. 1988; Baldo et al. 1989), *Cynodon dactylon* (Ford and Baldo 1986)

and *Artemisia princeps* (Park et al. 1989), have been studied for their IgE binding components.

According to the definitive study of the complete spectrum and rank order of allergens from *D. pteronyssinus* (Baldo et al. 1989), 32 fractions in the MW range of 6-140KD of the transferred proteins bound IgE antibodies in the sera from 96 patients allergic to house dust mites. The number of IgE-binding components in their study was greater than the number detected in any previous study of *D. pteronyssinus* (Lind and Löwenstein 1983; Le Mao et al. 1983; Krilis et al. 1984; Tovey and Baldo 1987). They proposed that these results might be explained by a much larger number of sera studies and recognition of the effects of reduction and blocking on IgE binding the process of identifying allergens by protein blotting.

The important components on the autoradiographs were bands with MW 95KD, 56KD, 54KD, 32KD, 30KD (Der p III), 26KD, 25KD (Der p I), 16KD (Der p II) and 15KD. Der p II exhibited the highest frequency of binding (88%) in 96 subjects. Der p I and Der p III have been identified in 43% and 68%, respectively. Five out of 96 sera showed monospecific recognition of Der p II.

In the study of IgE antibodies with specific radiolabelled allergen, Der f I (MW 24KD), Der f II (15KD) and Der f III (29KD) were identified in 78%, 94% and 16%, respectively, of 51 sera from mite-allergic patients (Heymann et al. 1989).

The relative clinical importance of the mite allergens has not yet been fully established. Group I and group II allergens have been studied and the study on group III allergens is ongoing (Platts-Mills and Chapman 1987; Heymann et al. 1989; Baldo et al. 1989).

Group I allergen (Der p I, Der f I) is a major protein component of extracts of fecal enriched culture material. The MW of Der p I has been variably reported as ranging from 24 to 30KD, and recently the isolation of a cDNA clone coding for Der p I has led to a derived MW of 25,371 for this allergen (Chua et al. 1988). Group II allergen (Der p II, Der f II) is the allergenic component of house dust mite body extract and has a MW close to 15KD on SDS-PAGE under nonreducing conditions.

In this study, the full spectrum of *D. farinae* extracts (whole body) on SDS-PAGE under nonreducing conditions was not excellent in separation of protein bands. However, all important allergenic components were there in position. On protein blotting there were 13 bands between 93KD and 12KD bound with patients' IgE. Tovey and Baldo

(1987) described the five most commonly and strongly bound components (MW 95, 53, 32 to 28, 25 and 15KD) of the body extract of *D. pteronyssinus*. Our result showed important components of 92-93KD(40.4%), 69-70KD(36.2%), 44-45KD (23.4%), 23-24KD (27.7%) and 14-15KD(95.7%) in the body extract of *D. farinae* when evaluated in 47 patients sensitive to house dust mites. The component at MW 14-15KD, which was suggestive as Der f II, bound IgE in 95.7% of 47 patients. Der f I, a MW 23-24KD component, bound in 27.7% and Der f III (28-29KD) bound in 19.1%. Therefore, the major allergenic component of *D. farinae* body extract is the component of MW 14-15KD, which is suggested as Der f II, in Korean allergic patients.

In Fig. 1, the pattern of SDS-PAGE of the extracts from Bencard materials of *D. pteronyssinus* and *D. farinae* was very similar to the pattern of feces enriched mite culture extracts, which was demonstrated by Tovey and Baldo(1987). On protein blotting, *D. pteronyssinus* extract showed 6 components bound to patients' IgE. The components of MW 14-15KD, 23-24KD and 28-29KD bound to IgE in 32.5 %, 22.5% and 22.5%, respectively, of 40 patients who responded strongly to house dust mites (RAST class 4 to both house dust mites).

Though it is difficult to directly compare our results to other reports, the binding % with group I allergen of *D. farinae* was very low in our data but the binding % with group III allergen of *D. farinae* (28-29KD) was similar to Heymann's result (Heymann *et al.* 1989). On protein blotting using the extract enriched with group I allergen of *D. pteronyssinus*, their results of binding % for the components of MW 28-29KD and 23-24KD were similar to our results of *D. farinae* components.

The different results of our data should be evaluated further according to several aspects, such as racial difference in immune response to house dust mites, house dust mites fauna in the house dusts and technical points.

Seven day exposure is enough for evaluation of most allergenic components by autoradiography. For the complete search of allergenic components, longer exposure may be needed. The comparison of IgE antibody responses to two or more allergenic components of the two house dust mites simultaneously may be a useful tool to evaluate individual or familial immune responses.

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