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Allergens of the house dust mite *Dermatophagoides farinae*—Immunochemical studies of four allergenic fractions

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Analysis of the allergenic components of the house dust mite Dermatophagoides farinae was performed. Four different types of proteins reactive with human anti-mite IgE antibodies were identified as four distinct radioprecipitins in radioimmunoelectrophoresis. These were designated as mite allergen reactive with human IgE antibodies (Me) Me 1, Me 2, Me 3, and Me 4 according to their electrophoretic mobility from the slower to the faster. Me 1 and Me 2 were the first and the second prevailing allergens, respectively, in 133 atopic patients subjected to the study. Me 1, on purification by gel filtration and anion exchange chromatography, elicited a single radioprecipitin by radioimmunoelectrophoresis. Me 1 also elicited a single band in sodium dodecyl sulphate-polyacrylamide gel electrophoresis and a single peak in high-performance liquid chromatography. Both methods elicited identical values of 17,000 daltons for the molecular weight of Me 1. The isoelectric point of Me 1 was suggested to be 8.0. Me 1 elicited higher skin reactions compared to that obtained by the crude mite extract when it was assessed in seven atopic patients by serial titration intradermal skin testing. (J ALLERGY CLIN IMMUNOL 75:686-92, 1985.)

The mite was suggested many years ago to be one of the major causative allergens found in house dust that is well known for its allergenicity in atopic asthma and other atopic diseases.^{1,2} More recently, the skin reaction to the extract of the pyroglyphoid mite of the *Dermatophagoides* sp has been reported to correlate well with that induced by house dust in atopic indi-

viduals,³⁻⁵ whereas others have found correlations in RAST and in provocation tests.^{6,7} We also previously reached the conclusion that the house dust mite was the most common source of allergen found in house dust in Japan⁸⁻¹¹ as it was for a large portion of Europe¹²⁻¹⁵ and the west coast of the United States.¹⁶

We have attempted to purify and characterize the allergenic components of the house dust mite *Dermatophagoides farinae* by use of a variety of immunochemical methods and have reported that the most allergenic fraction was distributed in the range of 20,000 to 30,000 daltons in molecular weight and that the major protein component was accompanied by a small amount of polysaccharide.^{17,18} We have made further attempts in this study to identify the component most allergenic to human atopic individuals by use of RIEP. This method has the advantage of directly iden-

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Received for publication April 10, 1984.

Accepted for publication Oct. 2, 1984.

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Abbreviations used

RIEP:	Radioimmuno-electrophoresis
DE-52:	Diethylaminoethyl cellulose
SDS-PAGE:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
PAG-IEF:	Polyacrylamide gel-isoelectrofocusing
HPLC:	High-performance liquid chromatography
BBS:	Borate buffered saline
PB:	Phosphate buffer
Rf:	Relative mobility
Me:	Mite allergen reactive with human IgE antibodies

tifying the mite antigen reactive with the patients' sera as well as the class of immunoglobulin with which they react. RIEP is technically much more convenient than crossed radioimmuno-electrophoresis and needs no anti-mite hyperimmune rabbit serum to bind mite proteins. The data presented here will demonstrate that there are at least four different types of allergenic molecules in the house dust mite reactive with patient IgE antibodies. Determination of the molecular weight of the major allergen fraction was attempted by HPLC and SDS-PAGE. The allergenicity of this fraction was confirmed by the intradermal skin test.

MATERIAL AND METHODS

Patients' sera

Sera from mite-allergic patients were selected at random from four sources: (1) 53 patients were selected from the outpatients' clinic of the Department of Medicine and Physical Therapy, Tokyo University, (2) 10 patients were selected from the Department of Dermatology, Tokyo Metropolitan Komagome Hospital, (3) 37 patients were selected from the Shinbashi Allergy Clinic, Shinbashi, Tokyo, and (4) 33 patients were selected from the Kameari Allergy Clinic, Kameari, Tokyo. Among the total of 133 patients selected, 64 patients were diagnosed as having bronchial asthma, whereas 49 patients were diagnosed as having a combination of bronchial asthma and atopic dermatitis. The remaining 13 patients were diagnosed as having atopic dermatitis, and another seven patients were diagnosed as having allergic rhinitis. All patients demonstrated positive skin reactions to the house dust mite but were examined for their mite specific IgE antibodies by the RAST method to confirm allergenicity to the house dust mite.

Mite allergen

Crude extract of the house dust mite (*D. farinae*) was prepared by the method previously described^{8,9} and kindly supplied by Torii & Co., Ltd., Tokyo, Japan. Briefly, the cultured mites were defatted with ether, and then 30 ml of PB was added to 1 gm of defatted mite and homogenized in an ice bath. Seventy milliliters of PB was added, and the

mixture was then stirred or rotated for 1 or 2 days. The mixture was centrifuged, and the supernatant was removed and dialyzed in distilled water for 48 hr, after which it was lyophilized. Two hundred milligrams of the lyophilized crude mite was solubilized in 5 ml of BBS (0.005 mol/L pH 8.0) and dialyzed against 5 L of BBS for 48 hr with one change, after which it was centrifuged at $10,000 \times g$ for 50 min. The supernatant was removed and stored at -20°C until use.

RAST was performed as previously described.⁷ The result was expressed in relation to a reference serum (H. I) that was granted arbitrary content of 1,000 pro mille (‰).

Intradermal skin test

Seven allergic patients were subjected to skin testing. Crude extract and the purified fraction or the DE-52 fraction I were diluted by BBS so that their optical density at 280 nm received 0.1. This has been confirmed to contain 0.1 mg of total solid in 1 ml of the solution in the case of the crude mite extract. Fivefold serial dilutions of these samples were prepared and used within 48 hr. Intradermal skin test was performed by the injection of 0.02 ml of 5^3 to 5^{12} dilution of each sample. Saline (0.9%) was used for controls. The longest and orthogonal diameters of the erythema and indurations were measured after 15 min.

RIEP

Titan IV IE plates (Helena Laboratories, Beaumont, Texas) were used as the agar plates in RIEP. Sodium barbital-barbital buffer (Daiichi Pure Chemical Co., Ltd., Tokyo, Japan) at an ionic strength of $\mu = 0.05$ (pH 8.6) was used as the running buffer. Ten microliters of the mite extract at a concentration of 0.1 mg/ml was applied to each well on the agar plate, followed by electrophoresis for 50 min at 4°C under a constant current of 40 mA per plate. Two hundred microliters of the patient's serum was subsequently applied to each corresponding trough to react with the migrated mite proteins. The agar plate was then kept in a humidity chamber for 24 hr at 4°C , followed by repeated washing with saline for 72 hr. One hundred microliters of ^{125}I -labeled anti-IgE antibody (5 $\mu\text{l}/\text{ml}$, 6,000,000 cpm/100 μl) was then applied to each trough to label the human anti-mite IgE antibodies. Antiserum against the Fc portion of E myeloma protein PS was raised in rabbits by the method previously described by Ishizaka et al.¹⁹ and was radiolabeled with ^{125}I (New England Nuclear, Boston, Mass.) as described by Klinman and Taylor.²⁰ After 48 hr of incubation in the humidity chamber, the plate was washed again for another 72 hr to wash away the excess radioactivity. The agar plate was then air dried to a thin film and was applied to a sheet of an x-ray film (Sakura x-ray film, new Y type, Konishiroku Photography Engineering Co. Ltd., Tokyo, Japan). Exposure time was varied from 24 hr to 120 hr depending on the radioactivity of the ^{125}I -labeled anti-IgE antibody. A serum (H. Y.) with a high titer of anti-mite IgE antibody was included in every agar plate as a control. Radiostaining on the x-ray film was measured by a densitometer (Canalco Co., Bethesda, Md.) and scored as fol-

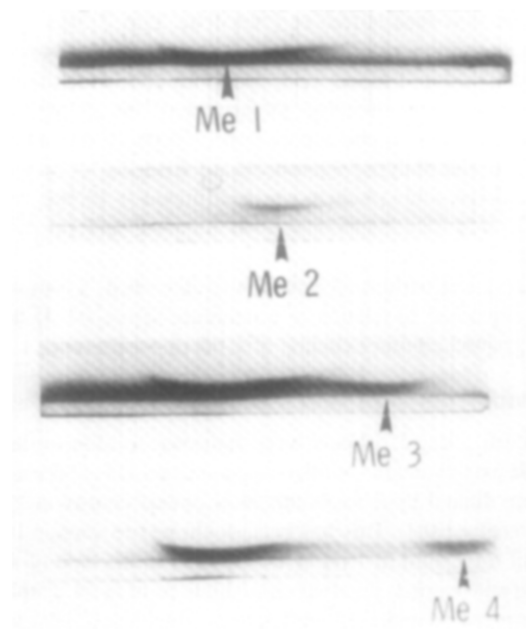


FIG. 1. Representative radioprecipitin bands by RIEP. Four different types of allergens were identified and designated Me 1, Me 2, Me 3, and Me 4 according to the electrophoretic mobility from the slower to the faster. Anode is to the right.

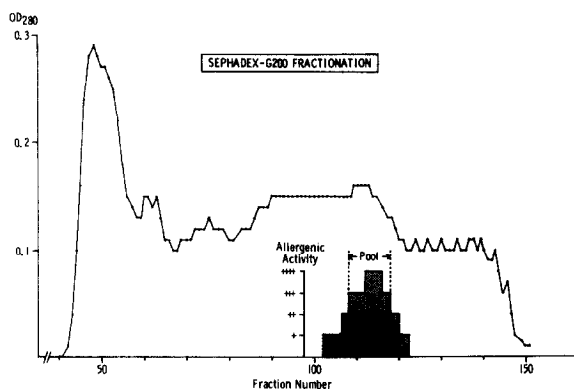


FIG. 2. Elution pattern of the crude mite extract. Three milliliters (40 mg/ml) of crude mite extract was applied to a Sephadex G-200 column (26 by 950 mm). The fraction volume was 3.8 ml. Allergenic activity determined by intensities of radiostaining of Me 1 is illustrated by the shaded area.

lows: + + +, absorbance of more than 80% of the radiostaining of the precipitin band formed by the control serum (H. Y.); + +, absorbance of between 50% to 80%; +, absorbance of between 30% to 50%; ±, absorbance of between 20% to 30%; and -, absorbance of less than 20%.

HPLC

A liquid chromatograph equipped with a type 45 pump, type USK, and type 440 universal injector absorbance de-

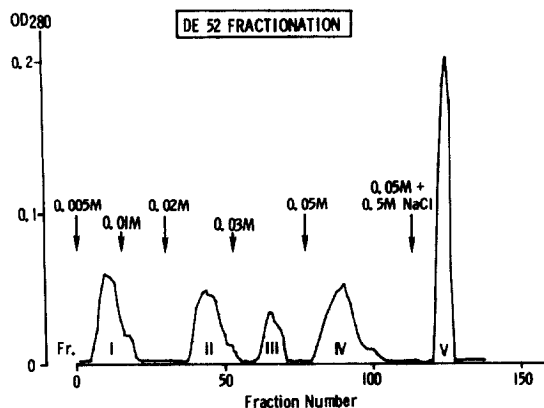


FIG. 3. Sephadex fractions of mite allergens 112 to 130 eluted from Sephadex G-75 column were pooled, concentrated, applied to a DE-52 ion exchange chromatography column (15 by 85 mm), and eluted with PB (pH 8.0). The concentrations of the PB were changed stepwise from 0.005 mol/L to 0.01, 0.02, 0.03, and 0.05 mol/L. Final elution was carried out with 0.05 mol/L of PB supplemented with 0.5 mol/L NaCl. Fraction volume was 5 ml.

tector were purchased from Waters Associates, Milford, Mass. The columns used were protein I-125 (7.8 by 300 mm; protein fraction range: 2,000 to 80,000 daltons) connected with protein I-250 (7.8 by 300 mm; protein fraction range: 10,000 to 50,000 daltons). The molecular weight determination was carried out by comparing the elution volumes of the marker proteins and those of the protein samples. The elution buffer was 1/15 mol/L of PB (pH 6.8), and the elution speed was 1.0 ml per minute. The concentration of the marker proteins and the protein samples were adjusted to 2 mg/ml, 25 μ l (50 μ g) of which was loaded on the column.

SDS-PAGE

SDS-PAGE was carried out following the method of Laemmli.²¹ Fifteen percent polyacrylamide gel was used in the present study. Twenty microliters of the crude mite (400 μ g), the purified mite (DE-52 fraction I, 40 μ g), or the marker protein was dissolved in 100 μ l of 0.0625 mol/L Tris-HCl (pH 6.8) containing 2% SDS and 10% glycerol, placed in boiling water for 3 min in the presence or absence of 1% 2-mercaptoethanol, and was allowed to cool before application to SDS-PAGE. Ten microliters of 0.05% bromophenol blue was added to the protein solution to mark the electrophoretic front. A total of 100 μ l of each sample was placed in the well, and electrophoresis was carried out for 2 hr at 3 mA per gel. Proteins on the gels were stained overnight with 0.25% Coomassie brilliant blue R-250 and destained with ethanol, acetic acid, and water (25:8:65). Rf of the marker proteins (low-molecular-weight electrophoresis calibration kit, Pharmacia Diagnostics, Uppsala, Sweden) against the electrophoretic front was calculated, and the molecular weight calibration curve was prepared.

PAG-IEF was carried out according to the method of Wrigley.²² Briefly, the gel consisting of 2% ampholine (pH

TABLE I. Radioimmuno-electrophoresis of sera of mite-sensitive patients

Radioprecipitin lines	Frequency of detection* (%)	Intensity of radiostaining (%)			
		+++	++	+	±
Me 1	100/133 (75.2)	28/133 (21.1)	15/133 (11.2)	37/133 (27.8)	20/133 (15.4)
Me 2	77/133 (56.4)	7/133 (5.3)	21/133 (15.8)	37/133 (27.8)	12/133 (9.0)
Me 3	19/133 (14.3)	2/133 (1.5)	3/133 (2.3)	7/133 (5.3)	7/133 (5.3)
Me 4	2/133 (0.5)	2/133 (1.5)	0/133 (0.0)	0/133 (0.0)	0/133 (0.0)

*103 of 133 sera of mite-sensitive patients demonstrated significant radioprecipitin lines of any kind.

3.5 to 10; LKB, Bromma, Sweden), 2.5% bis, and 7.5% acrylamide was photopolymerized by riboflavin in a glass tube (5 by 100 mm), and protein samples (2 mg/ml, 50 μ l) solubilized with 30% glycerol in 2% ampholine were applied. The electrophoresis was carried out for 3 to 4 hr at 200 V. The pI gradient was determined by measuring the pI after slicing and extracting the gels at 5 mm intervals. The proteins on the gels were fixed with 25% trichloroacetic acid, and the ampholine was removed by 25% ethanol and 8% acetic acid. The proteins were stained with 0.1% Coomassie brilliant blue R-250. The excess dye was then washed away with 25% ethanol and 8% acetic acid. The Rf values of the allergens were determined to estimate the pI of the allergens.

RESULTS

Immunologic heterogeneity of the mite allergen reactive with human anti-mite IgE antibody

RIEP to detect the mite allergen(s) reactive with human anti-mite IgE antibodies was carried out with sera from 133 atopic patients. Four distinct radioprecipitin bands were detected, indicating the presence of at least four different groups of allergen molecules reactive with human IgE antibodies. Such allergens were designated as Me 1, Me 2, Me 3, and Me 4 according to their electrophoretic mobility from the slower to the faster. The radioprecipitin bands, Me 1 to Me 4 observed in RIEP of four different representative sera of patients, is illustrated in Fig. 1. A summary of the RIEP patterns on reaction with sera of 133 atopic patients is illustrated in Table I. Radioprecipitin bands were observed in 100% (65/65), 73% (33/45), and 4% (1/23) of the atopic sera that demonstrated RAST titers more than 100%, 10% to 100%, and less than 10%, respectively. One hundred of 133 atopic sera (75%) demonstrated anti-Me 1 antibody, which corresponded to 97% of 103 positive for radioprecipitin lines of any kind. The strongly reactive radioprecipitin bands (+++) for Me 1 were found in 21.1% of the atopic sera, whereas only 5.3%, 1.5%, and 1.5% were strongly reactive with Me 2, Me 3, and Me 4, respectively. Me 2 was considered

to be the second major allergen judging from the frequency of detection and the intensities of the radioprecipitin bands. Correlation between the densities of Me 1 precipitation lines, those measured by densitometer, and RAST titers was examined. Correlation coefficient (r) was 0.75 and was statistically significant ($p < 0.001$).

Fractionation of the crude mite extract and molecular weight determination of Me 1, the major allergen fraction

Three milliliters of the crude mite extract solution (40 mg/ml) was applied to a Sephadex G-200 column (26 by 950 mm), and 3.8 ml each of the eluates was collected. The fractions were measured for optical densities at 280 nm and subjected to RIEP. Patient serum (H. Y.) used for the RIEP contained IgE antibodies to Me 1, Me 2, and Me 3. The most intensive radioprecipitin bands of Me 1 and Me 3 were concomitantly observed in fraction 112 (Fig. 2). Fractions 108 to 118 were pooled, concentrated to 3 ml, dialyzed against BBS, and applied to a Sephadex G-75 column (26 by 950 mm). Each eluate was measured for optical densities at 280 nm. The peak optical density was observed in fraction 121. Fractions 112 to 130 were pooled, concentrated to 5 ml (optical density 280 nm: 0.57), designated as the "mite-allergen Sephadex fraction," and subjected to further purification.

Four milliliters of the mite-allergen Sephadex fraction was dialyzed against 0.005 mol/L of PB (pH 8.0), applied to a DE-52 column (15 by 85 mm), and eluted with 0.005 mol/L of PB (pH 8.0). The concentration of PB was changed stepwise to 0.01 mol/L, 0.02, 0.03, and 0.05 mol/L. The final elution was carried out with 0.05 mol/L of PB supplemented with 0.5 mol/L NaCl. The fraction volume was 5 ml in each case. The elution profile is illustrated in Fig. 3. Five protein peaks obtained were named fractions I, II, III, IV, and V in order of their appearance, adjusted to 0.1 by optical density at 280 nm, and were subjected to RIEP. Equal amounts of sera from two atopic patients (H. Y. and Y. A.) were mixed and used for the

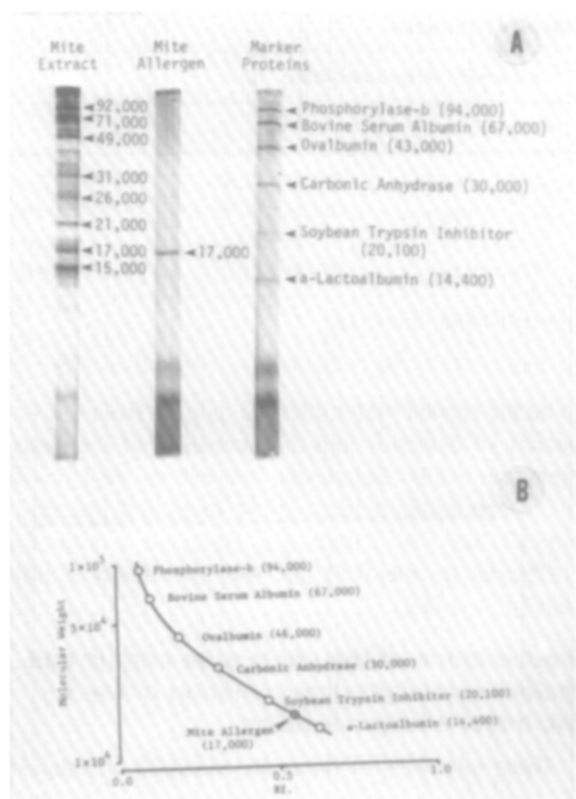


FIG. 4. Molecular weight determination of DE-52 fraction I by SDS-PAGE.

A, Electrophoretic patterns of the crude mite extract (left), DE-52 fraction I (center), and marker proteins (right).
B, Molecular weight calibration curve.

RIEP to develop radioprecipitin bands of Me 1, Me 2, Me 3, and Me 4, since serum from H. Y. contained IgE antibodies to Me 1, Me 2, and Me 3, whereas serum from Y. A. had IgE antibodies to Me 1 and Me 4. Clear radioprecipitin bands corresponding to Me 1 and Me 2 were observed in fractions I and II, respectively. Fraction III was a mixture of Me 2 and Me 3. Fraction IV appeared to contain small amounts of Me 3 and Me 4. Fraction V demonstrated a faint band of Me 3.

Since the DE-52 fraction I contained Me 1, this fraction was subjected to molecular weight analysis with HPLC. DE-52 fraction I demonstrated a narrow peak, which corresponded to a molecular weight of 17,000 daltons.

Subsequently, crude mite extract DE-52 fraction I was subjected to SDS-PAGE (Fig. 4). More than 20 protein bands were observed in the crude mite extract, whereas a single band appeared with DE-52 fraction I. The molecular weight of the Me 1 was also estimated to be 17,000 daltons. This molecular weight of the Me 1 fraction did not change after reduction with 2-mercaptoethanol.

TABLE II. Intradermal skin test with mite allergen fractions

Case	End points for each allergen*	
	Crude mite extract	DE-52 fraction I
M. H.	5^{-4}	5^{-7}
H. T.	5^{-4}	5^{-5}
K. K.	5^{-5}	5^{-6}
T. M.	5^{-3}	5^{-6}
C. Y.	5^{-9}	5^{-11}
Y. T.	5^{-3}	5^{-5}
K. H.	5^{-5}	5^{-9}
Geometric mean	0.32×5^{-4}	1.0×5^{-7}

*Final dilution of mite allergen fraction that elicited erythema more than 10 by 10 mm.

Determination of isoelectric points of mite allergens

DE-52 fractions were subjected to PAG-IEF. Fraction I elicited one clear protein band, and its pI was determined to be 8.0. Fractions II to IV each demonstrated three protein bands with pI values of 4.8, 5.4, and 6.0. Fraction V was a mixture of obscure multiple protein bands with different isoelectric points.

Intradermal skin test

Seven patients highly sensitive to mite by RAST and with positive RIEP were subjected to intradermal skin test to examine the biologic allergenicities of crude mite extract and DE-52 fraction I. The results are illustrated in Table II. End points for crude mite extract and for DE-52 fraction I correlated significantly ($\gamma = 0.86$; $p < 0.01$). Geometric means for crude mite extract and for DE-52 fraction I were 0.32×5^{-4} and 1.0×5^{-7} , respectively. About 40-fold increase of allergenic activity was observed for DE-52 fraction I compared to crude mite extract.

DISCUSSION

The data in the present study clearly demonstrate the heterogeneity of mite allergens and of anti-mite IgE antibodies in the sera of atopic patients. At least four types of mite protein with different electrophoretic mobilities reactive with anti-mite IgE antibodies were identified and designated Me 1, Me 2, Me 3, and Me 4. Me 1 was the most prevalent allergen. It generally formed intense radioprecipitin lines and was concluded to contain or to be the major allergen. End points for crude mite extract and for DE-52 fraction I (Me 1) correlated well ($\gamma = 0.86$; $p < 0.01$). Good correlation was also observed between the end points of skin test and RAST titers ($\gamma = 0.85$; $p < 0.01$).

Me 2 was considered to be the second major allergen from the frequency of detection and intensity of the radioprecipitin bands. Antigenic determinants of Me 1, Me 2, Me 3, and Me 4 appeared to be different, since the IgE antibodies to these allergens were observed independently. Heterogeneity of the mite allergens has been reported by several authors.²³⁻²⁵ Df 11 and Df 6 described by Dandeu et al.²⁵ appear to correspond to Me 1 and Me 2, respectively, from their frequency of detection and their slow electrophoretic mobility.

The molecular weight of Me 1, eluted in fraction I of DE-52 ion exchange chromatography, was determined in the present study to be about 17,000 daltons by HPLC and by SDS-PAGE. Other workers reported that the most important mite allergen resided in fractions with a molecular weight range of 12,500 to 45,000 daltons.^{13-15, 26} More recently, the molecular weights of purified mite allergens were described. The reported molecular weight was 28,000 daltons for Df 11,²⁵ 24,000 daltons for the P₁ antigen,²⁷ 27,000 daltons for Dpt 12,²⁸ and 25,000 to 30,000 daltons for Dpt 42.²⁹ Df 11 was obtained from the extract of *D. farinae*, whereas the latter three allergens were derived from the extract of *D. pteronyssinus*. At present it is not yet clear whether the major allergens described by the different research groups are the same or not. At least P₁ antigen appeared to be different from Me 1 or Df 11, since it had faster electrophoretic mobility. The PI values of the two peaks of P₁ antigen were 4.7 to 5.4 and 6.6 to 7.1,²⁷ whereas the pI value of Me 1 was 8.0. Such differences are reasonable because Le Mao et al.³⁰ reported that major allergens of *D. farinae* (Df 11) was antigenically distinct from that of *D. pteronyssinus* (P₁). Although similar in many of the characteristics, a discrepancy is observed between the molecular weight of Me 1 and Df 11, both derived from *D. farinae*. This remains to be explained. The difference in the method of extraction and/or the purification procedure may have caused such a difference. Careful comparison and establishment of standardized methods of extraction, purification, and detection appear necessary for future purification and characterization of the house dust mite allergens.

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Toxic oxygen products alter calcium homeostasis in an asthma model

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After anaphylactic or synthetic leukotriene C₄ contractions in guinea pig trachealis muscle, an accelerated initial rate and greater total myorelaxation are induced in these muscle preparations when they are immersed in calcium-free medium, O(Ca⁺⁺)_E. Inhibition of the late phase of anaphylaxis (ANA) by FPL 55712 (10⁻⁵ mol/L) eliminated the post-ANA O(Ca⁺⁺)_E-augmented myorelaxation, suggesting a causal role for SRS-A products. Hypoxia or superoxide dismutase/catalase pretreatment also abolished the post-ANA or leukotriene C₄ O(Ca⁺⁺)_E-augmented myorelaxation. The data support the hypothesis that toxic oxygen products generated with SRS-A and/or LTC₄ induce an alteration in Ca⁺⁺ homeostasis in airway smooth muscle. In this model of allergic asthma, airway smooth muscle alteration after ANA may contribute to the pathogenesis of asthma and/or airway hypersensitivity associated with allergic asthma. (J ALLERGY CLIN IMMUNOL 75:692-97, 1985.)

One possible cause of asthma is a primary defect of ASM. Previous work from this and other laboratories indicated an acquired abnormality of calcium homeostasis in ASM hyperreactivity.^{1,2} We found an increased sensitivity of RIT to extracellular calcium (Ca⁺⁺)_E after in vitro ANA in guinea pig trachealis.¹ In this study subphysiologic extracellular calcium conditions were required to distinguish an effect on RIT in contrast to previous investigations of experimental asthma in which ASM hyperreactivity was analyzed by agonist-contraction responses.³ Additionally, the

Abbreviations used

ANA:	Anaphylaxis
LT:	Leukotriene
O(Ca ⁺⁺) _E :	Zero calcium
SOD:	Superoxide dismutase
CAT:	Catalase
ASM:	Airway smooth muscle
RIT:	Resting isometric tension
KH:	Krebs-Henseleit
Ri:	Initial velocity of relaxation
Rm:	Maximal relaxation

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Received for publication June 1, 1984.

Accepted for publication Oct. 3, 1984.

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potential contribution of resting basal muscle tone to airway hyperreactivity has been questioned.⁴ During our experiments we observed that the initial rate and total magnitude of relaxation of RIT in a calcium-free medium (O[Ca⁺⁺]_E) was enhanced after anaphylaxis (Fig. 1, A). This article validates this finding and also