



CLONING AND CHARACTERIZATION OF A NEW ALLERGEN, Mag 3, FROM THE HOUSE DUST MITE, *DERMATOPHAGOIDES FARINAE*: CROSS-REACTIVITY WITH HIGH-MOLECULAR-WEIGHT ALLERGEN

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Abstract—A new immunoreactive clone whose sequence is not homologous to that of any of the previously identified mite allergens was isolated by successive immunoscreening of a *Dermatophagoides farinae* cDNA library with rabbit antisera to an extract of the house dust mite and IgE in pooled sera from patients allergic to mites. Rabbit antibodies specific for the recombinant protein recognized a 177 kD protein in a mite body extract. This immunoreactive protein was located in the circumferential tissues of esophagus, gut and the other internal organs in mites. The reaction of human IgE to the purified natural antigen was inhibited competitively to 30% by the recombinant antigen. In terms of the frequency and the intensity of response to specific IgE in sera from asthmatic patients, the natural protein was similar to *Der f* 2, while the recombinant protein was slightly less allergenic by these criteria. We conclude that the natural protein from the house dust mite, *D. farinae*, is an important allergen. Copyright © 1996 Elsevier Science Ltd.

Key words: *Dermatophagoides farinae*, recombinant allergen, cross-reactivity, high-molecular-weight allergen.

INTRODUCTION

Since house dust mites (*Dermatophagoides* sp.) were reported as one of the major agents causing allergic diseases such as bronchial asthma, perennial rhinitis and atopic dermatitis (Voohorst *et al.*, 1967), many efforts have been made to identify allergens for humans from two closely related mite species, *D. farinae* and *D. pteronyssinus* (Arlian, 1991; Platts-Mills *et al.*, 1992).

A large number of allergenic components has been identified in extracts of mite culture, although the exact numbers of reported components varies from 4 to 26 and from 5 to 19 in mite bodies and feces, respectively (Arlian *et al.*, 1987a, 1987b). Among them, antigens in groups of *Der* 1 (25 kD), *Der* 2 (14 kD), *Der* 3 (28/30 kD), *Der* 4 (60 kD), *Der* 5 (14 kD), *Der* 6 (25 kD) and *Der* 7 (22–28 kD) have been characterized as major or important allergens (King *et al.*, 1994). We have found and identified

new *D. farinae* antigens, such as Mag 1 (39 kD), Mag 29 (68 kD) and Mag 44 (37 kD) as the corresponding allergens (Aki *et al.*, 1994a, 1994b, 1994c, 1995) and additional low-molecular-weight antigens which appear to be asthma-inducible (Yamaguchi *et al.*, 1994a, 1994b). Mag 29 is nearly identical in sequence to heat shock protein 70, while Mag 44 has been identified as tropomyosin. Recently, another antigen which is highly homologous to glutathione S-transferase was reported to be a major mite allergen (O'Neil *et al.*, 1994).

Most of the major or important allergens in mite body and feces extracts are found in a molecular weight range of 10–70 kD. In some cases, larger allergens (> 110 kD, > 158 kD, 174 kD and 175 kD) could be detected by immunoelectrophoresis and/or immunoblotting, but they are not fully characterized (Stewart and Turner, 1980a; Baldo *et al.*, 1989; Wahl *et al.*, 1989; Colloff *et al.*, 1992). We have found that high-molecular-weight antigens isolated in our laboratory are mitogenic allergens (Shigeta *et al.*, 1995). However, much work remains to be done in characterizing all potentially allergenic antigens.

In this paper, we report the molecular cloning of a cDNA encoding a new allergenic component named Mag 3 from a house dust mite *D. farinae* cDNA library. We

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Abbreviations: Dfb, mite body extract from *Dermatophagoides farinae*; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thio-galactopyranoside; *mag*, cDNA coding for recombinant mite antigen; PBS, phosphate-buffered saline.

found also that the polypeptide produced by the cDNA clone shares antigenic determinants with natural high-molecular-weight allergen that possesses the frequency and capacity of IgE-binding comparable to the major allergen, *Der f 2*.

MATERIALS AND METHODS

Antigens

Live mites, *D. farinae*, and the spent growth media were supplied by Fumakilla Ltd (Hiroshima, Japan). Mite body extract (Dfb) was prepared in the presence of protease inhibitors [0.1 mM phenylmethylsulfonyl fluoride, 1 mM monoiodoacetic acid, 5 mM 1,2-epoxy-3-(*p*-nitrophenoxy)propane, and 5 mM EDTA] from a whole spent growth medium as described previously (Yamaguchi *et al.*, 1993). The major allergens, *Der f 2*, were kindly provided by Dr H. Yasueda of the National Sagami Hospital (Kanagawa, Japan).

Human sera and rabbit antisera

Mite-allergic sera were collected from 23 asthmatic patients who elicited a positive skin test (> 20 mm in mean diameter of erythema) to mite and house dust extracts (Torii & Co., Ltd, Tokyo, Japan). Four sera from healthy volunteers who provoked a negative skin reaction (< 9 mm) to both extracts were used as controls.

Rabbit anti-Dfb sera were used as prepared in our laboratory (Aki *et al.*, 1994c). Antisera were prepared by immunizing rabbits intramuscularly four times every 2 weeks with 100 µg of a recombinant antigen in Freund's complete adjuvant. Rabbit anti-*Der f 1* and anti-*Der f 2* sera were kindly provided by Dr H. Yasueda.

Construction and screening of D. farinae cDNA library

A cDNA clone coding for a new mite allergen was isolated according to the methods previously described (Aki *et al.*, 1994a). Briefly, poly(A)-mRNA was isolated from live mites with a Fast Track mRNA isolation kit (Invitrogen Co., San Diego, CA, U.S.A.) after guanidinium isothiocyanate extraction. A mite cDNA library was constructed in λgt11 (Stratagene, La Jolla, CA, U.S.A.) by using a cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden) and an *in vitro* packaging kit (Stratagene). After *E. coli* Y1090 was infected with the recombinant phages, positive lysates were immunoscreened with rabbit anti-Dfb serum.

Expression and purification of recombinant mite allergen

The cDNA encoding immunoreactive protein (Mag 3) was ligated to a plasmid pGEX-2T (Pharmacia Biotech). The resultant plasmid was transformed into *E. coli* JM109. The transformant was grown at 37°C in 2 l of 2xYT medium to OD₆₀₀ unit of 0.6. After IPTG induction for 3 hr, a fusion protein (GST-Mag 3) was recovered as inclusion bodies in cells thoroughly sonicated in 50 mM Tris-HCl pH 7.6 containing 5 mM EDTA, 0.1% 2-mercaptoethanol and 1% Triton X-100. The inclusion bodies

dissolved in sample buffer were applied onto a gel in an apparatus (model NA-1800, Nippon Eido Co., Tokyo, Japan) for preparative disk gel electrophoresis according to the manufacturer's directions. The target fusion protein isolated by the electrophoresis was cleaved with human thrombin (Sigma, St Louis, MO, U.S.A.) in 50 mM Tris-HCl, pH 8.0 (Smith and Johnson, 1988; Frangioni and Neel, 1993). Recombinant Mag 3 was purified from the digest by the electrophoresis. Finally, the purified recombinant Mag 3 was lyophilized after dialysis and stored at -80°C.

Isolation of natural antigen that shares antigenic determinants with the clone protein

Rabbit anti-recombinant Mag 3 serum (30 ml) diluted 10-fold with 20 mM phosphate-buffered saline (PBS) containing 0.5% Triton X-100 (PBST) was applied onto an immobilized recombinant Mag 3 column prepared using CNBr-activated Sepharose CL-4B (Pharmacia Biotech) and circulated overnight at 4°C. The affinity column was sufficiently washed with PBS, followed by elution of specific anti-recombinant Mag 3 antibody with 0.2 M glycine-HCl (pH 2.25) containing 150 mM NaCl. The eluates were neutralized immediately with 1 M Tris-HCl buffer (pH 8.0) and stored at -80°C until use. Two hundred milligrams of Dfb dissolved in PBST containing protease inhibitors was applied onto an immobilized specific anti-recombinant Mag 3 antibody Sepharose CL-4B. The natural antigen was recovered by the same procedures and preserved in PBS supplemented with 50% glycerol.

SDS-PAGE and immunoblotting

The SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970). The separated proteins were electrotransferred from the gel to an Immobilon-P membrane (Millipore Corporation, Bedford, MA, U.S.A.) using a semi-dry blotting apparatus and visualized by immunostaining (Tovey and Baldo, 1987c).

The levels of specific IgE antibody in mite-allergic sera

The levels of IgE antibody specific for recombinant Mag 3, natural antigen, Dfb and *Der f 2* were estimated by ELISA (Aki *et al.*, 1994b). In brief, an ELISA plate (Falcon 3912, Becton Dickinson & Co., Oxnard, CA, U.S.A.) was coated with recombinant Mag 3 (0.5 µg), natural antigen (1.8 µg), *Der f 2* (0.2 µg) and Dfb (10 µg), followed by blocking with 3% dextran in PBST. Bound IgE antibody was detected by means of sequential incubation with affinity-purified biotin-conjugated goat anti-human IgE (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, U.S.A.), affinity-purified peroxidase-conjugated streptavidin (Tago Inc., Burlingame, CA, U.S.A.) and 10 mM citrate buffer pH 4.2 containing 2,2'-azinobis(3-ethylbenzo-thiazoline-6-sulfate) and H₂O₂. Absorbance at 415 nm was measured. Simultaneously, an ELISA plate was coated with sheep anti-human IgE (Cappel, West Chester, PA, U.S.A.). After blocking, a series of diluted human IgE (Yamasa Shoyu Co., Ltd,

Chiba, Japan) was incubated. The bound IgE antibody was estimated by the above-mentioned procedures. The unit of specific IgE antibody in mite-allergic sera is defined as the concentration of IgE (ng/ml) required to produce the same ELISA value on standard curve for the binding of IgE by anti-human IgE.

ELISA inhibition

An ELISA plate was coated with natural antigen. After blocking with 3% dextran in PBST, mite-allergic sera or rabbit anti-Mag 3 sera were preincubated for 30 min at 37°C with serial dilutions of Mag 3, natural antigen, or cedar pollen extract. The treated sera were incubated in the blocked wells. The bound antibody was detected by the same procedures as above.

Nucleotide and amino acid sequencing

Nucleotide sequencing by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) was performed using an AutoRead Sequencing kit and A.L.F. DNA sequencer (Pharmacia Biotech). Amino acid sequence was determined by the automated Edman degradation method on a Protein Sequencer (model 473A, Applied Biosystems Japan Inc., Tokyo, Japan).

Immunohistochemical staining

Live mites were fixed overnight at 4°C in 37.5 mM phosphate buffer pH 6.2 containing 10 mM NaIO₄, 75 mM lysine and 2% paraformaldehyde. The fixed mites were dehydrated sequentially and dealcoholized with increasing concentrations of ethanol and xylene, respectively, followed by embedding in paraffin. Thin sections (4 µ) were adhered on silanized object glasses. After deparaffinization, internal peroxidase was inactivated by incubation with 3% H₂O₂ in methanol. The sections were overlaid with purified rabbit anti-Mag 3 antibody after blocking with PBST containing 10% goat normal serum (Dako A/S, Glostrup, Denmark). Peroxidase-conjugated goat anti-rabbit immunoglobulin (Cappel) and 3,3'-diaminobenzidine were used for the specific antibody binding in mite tissues. The preparations were observed with hand-stand type light microscope (IMT-2, Olympus Optical Co., Ltd, Tokyo, Japan) (Brody *et al.*, 1972; Tongu *et al.*, 1986).

RESULTS

Cloning and nucleotide sequencing of mite antigen cDNA

An immunoreactive clone was isolated from a *D. farinae* cDNA library by sequential screening with rabbit anti-Dfb serum and pooled IgE sera from patients allergic to mites. The nucleotide sequence of the clone, *mag 3*, was determined and the corresponding amino acid sequence (Mag 3) was deduced (Fig. 1). The cDNA, consisting of 1049 bp, codes for 349 amino acids corresponding to a molecular weight of 40,545. Neither an initiation codon nor a termination codon was, however, evident in the sequence. Furthermore, no homology between this

sequence or the derived amino acid sequence and other DNA sequences or proteins, respectively, was found by searching the sequence with those present in EMBL/GenBank/DBJ.

Expression and purification of the cloned protein

A fusion protein (GST-Mag 3) recovered as inclusion bodies after sonication of IPTG-induced transformant cells could not be dissolved under the mild condition tested. After solubilization with SDS, preparative disk gel electrophoresis was carried out to purify the fusion protein. The recombinant protein (Mag 3) was purified from a thrombin digest of the fusion protein by the same electrophoresis method. The yield of the recombinant Mag 3 was 8.5 mg from 2 l of the culture. The mobility of the GST-Mag 3 product on an SDS-PAGE corresponds to a molecular weight of 66 kD [Fig. 2(A), lane 2], while the purified recombinant Mag 3 migrated as a single band at a position corresponding to 42 kD (lane 4), slightly greater than the 40 kD predicted by the sequence.

Isolation of natural antigen that shares antigenic determinant with recombinant Mag 3

Two hundred micrograms of natural antigen that cross-reacted with recombinant Mag 3 was eluted from an immobilized anti-recombinant Mag 3 antibody column loaded with Dfb (200 mg). The purified natural antigen migrated on a SDS-polyacrylamide gel under reducing or non-reducing condition as an almost single band possessing a mobility corresponding to a molecular weight of 177 kD [Fig. 2(B), lanes 3 and 5], four times larger than the recombinant Mag 3. However, the purified antigen preparation contained minor immunoreactive bands in addition to a major 177 kD protein termed natural Mag 3 on a Western blot [Fig. 2(C), lane 2]. Specificity of these immunoreactive bands was confirmed by our finding that the binding activity of anti-recombinant Mag 3 to these components was inhibited completely by the purified recombinant Mag 3 (data not shown). This result indicated that these immunoreactive proteins share common epitopes with recombinant Mag 3.

*Internal localization of natural antigen in *D. farinae* by histochemical staining*

Although contraction of the section was observed in the cuticular layer of mites, the internal organization was well preserved. Anti-recombinant Mag 3 antibody bound to the circumferential tissues of esophagus, gut and the other internal organs [Fig. 3(A)], while normal rabbit serum did not [Fig. 3(B)]. This result demonstrates directly that natural antigen is mite-component.

The levels of IgE specific for recombinant Mag 3 and natural Mag 3 in mite-allergic sera

The levels of IgE specific for recombinant Mag 3 and natural Mag 3 in mite-allergic sera (23 cases) and healthy sera (four cases) were measured on ELISA in comparison

1	V	T	A	L	E	L	L	L	K	10	G	E	T	E	D	K	T	R	R	Y	20
A	GTG	ACC	GCA	TTG	GAA	TTA	TTG	TTG	AAA	GGT	GAA	ACT	GAA	GAC	AAA	ACT	CGT	CGC	TAT	GT	
										30	S	N	K	Q	A	K	A	Q	I	E	V
	A	E	L	T	A	V	G	S	P	S	AGC	AAC	AAA	CAA	GCT	AAA	GCA	CAG	ATC	GAA	GTG
	GCC	GAA	TTG	ACT	GCC	GTT	GGA	TCG	CCA	50	L	K	S	P	E	H	E	F	N	T	E
	T	K	G	E	E	Y	K	I	T	L	AAA	TCA	CCT	GAA	CAT	GAA	TTT	AAC	ACC	GAA	80
	ACC	AAA	GGC	GAA	GAA	TAC	AAA	ATC	ACA	70	L	K	M	H	M	D	F	P	N	V	F
	F	T	I	H	A	D	K	N	N	L	AAA	ATG	CAC	ATG	GAT	TTT	CCT	AAC	GTG	TTT	100
	TTC	ACC	ATC	CAT	GCA	GAC	AAA	AAC	AAT	90	L	K	M	H	M	D	F	P	N	V	F
	Q	A	D	L	T	G	T	F	Q	H	D	K	E	N	N	V	R	K	N	Q	120
	CAA	GCT	GAT	CTG	ACT	GGA	ACT	TTC	CAA	CAT	GAC	AAG	GAA	AAC	AAT	GTG	CGT	AAA	AAC	CAA	140
	L	N	L	Q	Y	K	F	A	G	D	E	K	P	H	T	V	D	Y	E	N	160
	TTG	AAT	CTC	CAA	TAC	AAA	TTC	GCT	GGT	GAT	GAG	AAA	CCA	CAC	ACA	GTG	GAT	TAT	GAA	AAC	180
	E	F	S	F	N	L	K	R	S	S	K	D	K	N	S	G	V	D	Y	R	200
	GAA	TTT	TCA	TTC	AAT	TTG	AAA	CGA	TCA	TCA	AAA	GAC	AAA	AAC	AGT	GGG	GTC	GAT	TAC	CGA	220
										150											160
	A	K	Y	M	S	S	H	F	P	I	L	N	H	K	V	N	I	Q	F	K	180
	GCA	AAA	TAC	ATG	TCT	AGC	CAT	TTT	CCA	ATC	TTG	AAC	CAC	AAA	GTC	AAC	ATT	CAA	TTT	CAA	200
	Y	R	P	F	K	V	N	E	L	N	L	E	G	E	F	G	R	E	L	Q	220
	TAT	CGA	CCA	TTC	AAA	GTT	AAC	GAA	TTG	AAT	TTG	GAA	GGA	GAA	TTT	GGT	CGA	GAA	CTT	CAA	240
	H	K	F	Q	L	M	R	N	S	Q	I	E	V	E	E	V	R	P	F	K	260
	CAC	AAA	TTC	CAA	TTG	ATG	CGA	AAC	AGC	CAG	ATA	GAA	GTG	GAA	GAA	GTG	CGA	CCA	TTT	AAG	280
	M	H	G	N	S	D	I	K	L	M	A	N	D	L	D	I	D	Y	D	L	300
	ATG	CAT	GGC	AAT	AGT	GAC	ATA	AAA	TTG	ATG	GCA	AAC	GAT	CTG	GAT	ATT	GAT	TAT	GAT	CTC	320
	K	S	E	F	K	Y	E	S	N	K	G	T	P	I	E	L	Q	Y	K	I	340
	AAA	TCT	GAA	TTC	AAA	TAT	GAA	AGC	AAC	AAA	GGA	ACC	CCC	ATT	GAA	TTG	CAA	TAC	AAA	ATT	360
	S	G	K	D	R	S	K	R	A	A	D	L	G	A	E	D	V	E	G	V	380
	TCC	GGT	AAA	GAT	CGC	TCG	AAA	AGA	GCT	GCC	GAT	TTG	GGA	GCC	GAA	GAT	GTT	GAA	GGC	GTT	400
	I	D	Y	K	N	N	G	S	P	I	D	S	K	M	H	A	H	L	K	M	420
	ATC	GAT	TAC	AAA	AAC	AAT	GGT	TCG	CCA	ATT	GAT	TCT	AAG	ATG	CAT	GCT	CAT	CTC	AAA	ATG	440
	K	G	N	N	Y	G	Y	D	S	E	L	K	Q	T	Q	P	Q	Q	Y	E	460
	AAA	GGC	AAC	AAC	TAT	GGC	TAT	GAT	AGT	GAA	TTG	AAA	CAA	ACA	CAG	CCA	CAA	CAA	TAT	GAA	480
	G	K	I	T	L	S	K	N	D	K	K	I	F	I	N	H	K	S	E	M	500
	GGC	AAA	ATC	ACA	TTG	TCC	AAG	AAT	GAC	AAG	AAA	ATC	TTC	ATC	AAC	CAC	AAA	TCA	GAG	ATG	520
	T	K	P	T	N	T	F	H	L	K	T	D	A	D	V	S	Y	S	D	S	540
	ACC	AAA	CCT	ACC	AAC	ACT	TTC	CAT	CTC	AAA	ACA	GAT	GCT	GAT	GTG	TCG	TAT	TCG	GAT	TCG	560
	D	M	K	K	H	Y	Q	M	E												580
	GAT	ATG	AAA	AAA	CAT	TAT	CAG	ATG	GAA	T											600

Fig. 1. Nucleotide sequence of *mag* 3. The deduced amino acid sequence and numbers are shown in middle and upper rows, respectively. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the following accession number D17686.

with those to Dfb and *Der f* 2 (Table 1). The IgE specific for these antigens in mite-allergic sera varied largely with the difference in individual sera. When more than cut-off values were regarded as significant responses, IgE from nine out of 23 patients (39%) bound to recombinant Mag 3, while IgE from 16 patients (70%) bound to natural Mag 3. These percentages were compatible with the proportion of these patients (13 out of 23; 57%) whose sera contained IgE specific for *Der f* 2. Moreover, the mean titer of IgE specific for natural Mag 3 was equal to or higher than the level of IgE directed to *Der f* 2.

The capacity of specific IgE binding to recombinant Mag 3 in sera from patients allergic to mites was com-

pared with that binding to natural Mag 3 (Fig. 4). Significant correlation was observed and the correlation coefficient was calculated to be 0.64. All dots from the patients who responded significantly to both antigens were located over the thick broken line.

Cross-reactivity of recombinant Mag 3 with natural Mag 3

Cross-reactivity of recombinant Mag 3 with natural Mag 3 was examined by ELISA inhibition for human pooled sera from subjects positive to both recombinant Mag 3 and natural Mag 3, and for rabbit anti-recom-

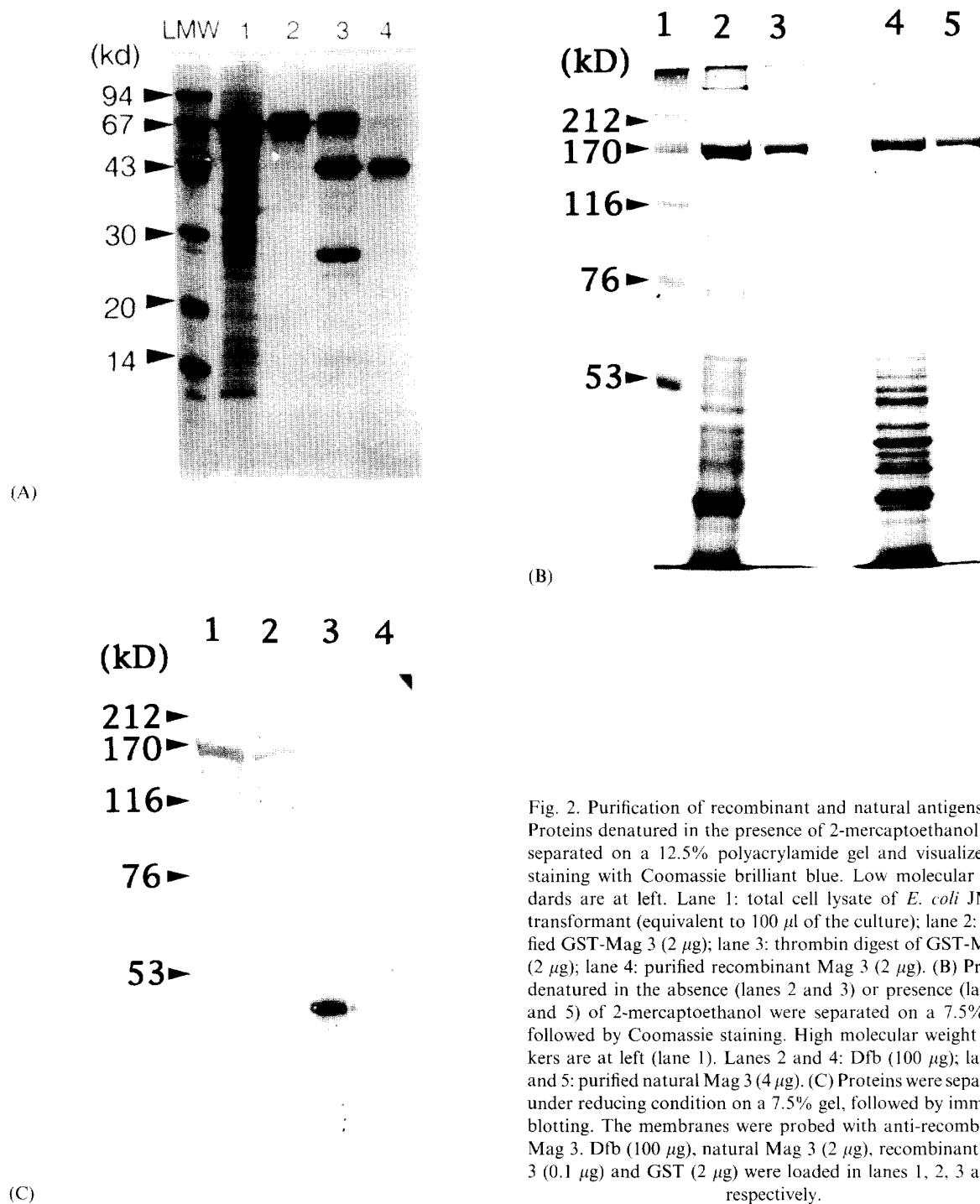


Fig. 2. Purification of recombinant and natural antigens. (A) Proteins denatured in the presence of 2-mercaptoethanol were separated on a 12.5% polyacrylamide gel and visualized by staining with Coomassie brilliant blue. Low molecular standards are at left. Lane 1: total cell lysate of *E. coli* JM109 transformant (equivalent to 100 µl of the culture); lane 2: purified GST-Mag 3 (2 µg); lane 3: thrombin digest of GST-Mag 3 (2 µg); lane 4: purified recombinant Mag 3 (2 µg). (B) Protein denatured in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of 2-mercaptoethanol were separated on a 7.5% gel, followed by Coomassie staining. High molecular weight markers are at left (lane 1). Lanes 2 and 4: Dfb (100 µg); lanes 3 and 5: purified natural Mag 3 (4 µg). (C) Proteins were separated under reducing condition on a 7.5% gel, followed by immunoblotting. The membranes were probed with anti-recombinant Mag 3. Dfb (100 µg), natural Mag 3 (2 µg), recombinant Mag 3 (0.1 µg) and GST (2 µg) were loaded in lanes 1, 2, 3 and 4, respectively.

binant Mag 3 serum (Fig. 5). The reaction of the human sera to natural Mag 3 was inhibited completely by the natural Mag 3, while recombinant Mag 3 suppressed the reaction to only 30%. This result suggests that natural Mag 3 should possess additional epitopes besides those of recombinant Mag 3. The reaction of rabbit anti-recombinant Mag 3 to natural Mag 3 was inhibited completely by both antigens.

DISCUSSION

A cDNA clone, *mag* 3, was a representative gene of a group among nine discrete groups, into which immuno-reactive clones were classified according to DNA cross-hybridization (Aki *et al.*, 1995). Although *mag* 3 codes for only a part of a certain protein, the gene expression product, recombinant Mag 3, had remarkable mitogenic

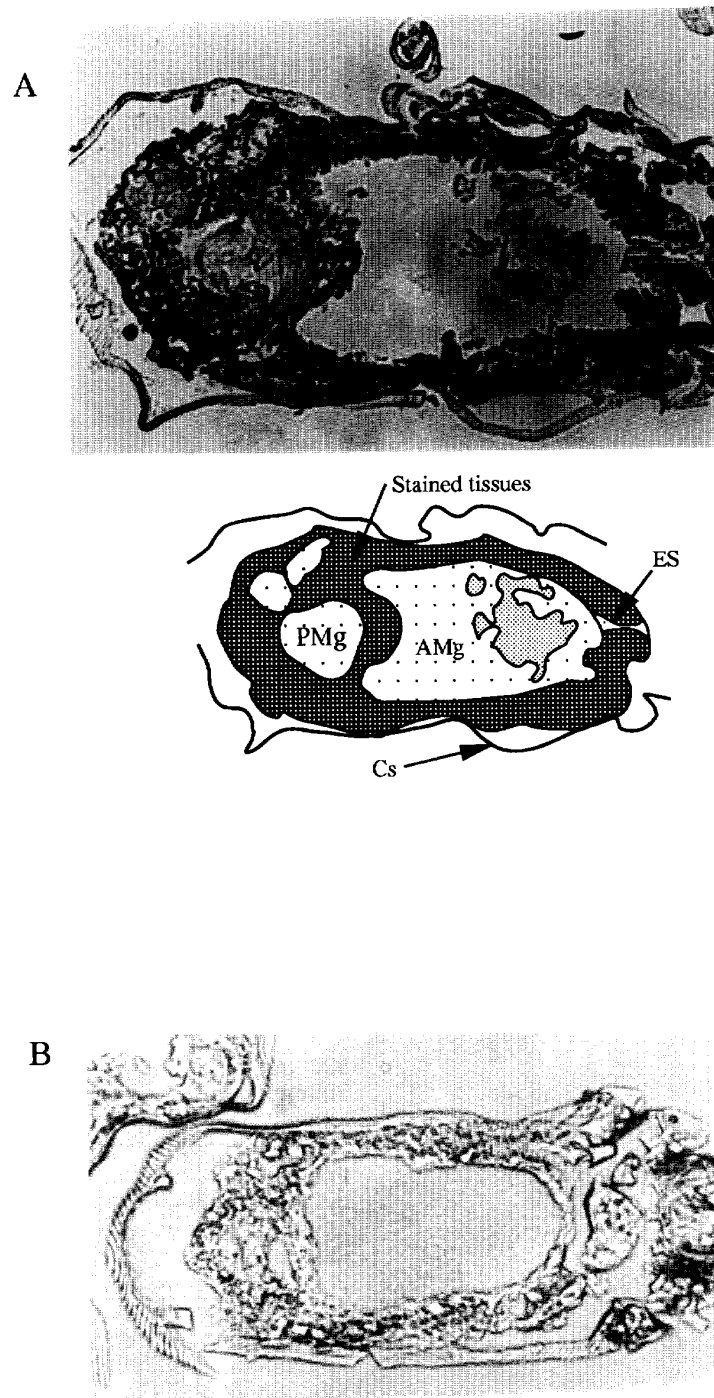


Fig. 3. Immunohistochemical staining of *D. farinae*. Symbols: Es, esophagus; AMg, anterior midgut; PMg, posterior midgut; Cs, cuticular layer of mite body surface.

activity on human and murine B cells (Fujii *et al.*, 1995). To elucidate the structure and function of natural protein, we carried out a homology search for *mag 3* and its deduced amino acid sequence, but no significant similarity to any other genes and proteins was found. Using an immobilized anti-recombinant Mag 3 column, we isolated a natural 177 kD protein which co-purified with some minor protein components. These minor com-

ponents appear to be degraded products of the major protein as they were not present in some other preparations of live mites, and their levels generally increased when mite extracts were preserved in the absence of protease inhibitors (data not shown).

The staining of the natural Mag 3 with periodate–Schiff's reagent and Sudan black B was under the limit of detection when 50 μ g of the protein was used (data not

Table 1. The levels of IgE specific for respective antigens in sera from patients allergic to mites and sera from healthy donors

Case	Dfb	IgE antibody, units		<i>Der f</i> 2
		rMag 3 ^a	nMag 3	
1	14.97	5.71	10.07	< 0.02
2	14.10	8.26	8.42	< 0.02
3	12.11	5.32	7.00	< 0.02
4	9.59	2.60	3.32	11.20
5	9.42	1.05	2.91	0.13
6	9.02	2.13	4.31	< 0.02
7	8.85	1.56	2.22	1.74
8	8.49	< 0.02	5.66	17.49
9	7.75	3.30	6.93	9.61
10	5.50	3.90	9.66	14.58
11	5.25	1.61	1.07	6.54
12	4.90	1.06	1.50	3.27
13	4.48	1.38	5.88	6.06
14	3.16	< 0.02	0.02	0.70
15	3.12	0.28	< 0.02	7.58
16	2.84	0.53	8.09	2.45
17	2.77	4.02	5.78	4.91
18	2.42	1.50	3.36	3.94
19	2.24	1.06	0.35	0.86
20	0.92	1.61	1.32	0.67
21	0.89	1.83	7.23	0.83
22	0.87	2.23	5.58	< 0.02
23	< 0.02	1.40	3.04	1.49
24	< 0.02	< 0.02	< 0.02	< 0.02
25	< 0.02	< 0.02	< 0.02	< 0.02
26	< 0.02	< 0.02	< 0.02	< 0.02
27	< 0.02	< 0.02	< 0.02	< 0.02
MS	5.74	2.23	4.50	4.07
CO	1.95	2.12	2.23	1.38
N	19	9	16	13

^aSymbols: rMag 3, recombinant Mag 3; nMag 3, natural Mag 3; MS, mean of the IgE levels; CO, cut-off value; N, numbers of patients having a significant IgE titer. Cut-off values were determined by adding the mean + 3 SD of the differences between the measured value and the average one in individual serum to the mean IgE level (~0 unit) of healthy donors. Cases 1–23 and 24–27 were sera from mite-allergic patients and healthy volunteers (controls), respectively.

shown). Thus, the natural Mag 3 is not the 174 kD antigen reported previously (Stewart and Turner, 1980a) or the 150–155 kD allergens which have been found in our laboratory (Shigeta *et al.*, 1995), because of their high saccharide contents. Similarly, the Dpt 4, which has been described as a lipoprotein of 244 kD or 274 kD (Stewart and Turner, 1980a, 1980b; Stewart *et al.*, 1983), is not identical to the natural Mag 3 in terms of its molecular weight and lipid content. The natural Mag 3 also is not the 170 kD antigen reported previously (Abe and Ishii, 1982) because that protein was composed of several subunits. Natural Mag 3 (10 µg) and recombinant Mag 3 (10 µg) did not react with anti-*Der f* 1 and anti-*Der f* 2 on immunoblots (data not shown). Furthermore, allergenicity of natural Mag 3 and recombinant Mag 3 was demonstrated by *in vivo* skin tests and/or histamine release assay (data not shown). These results suggested

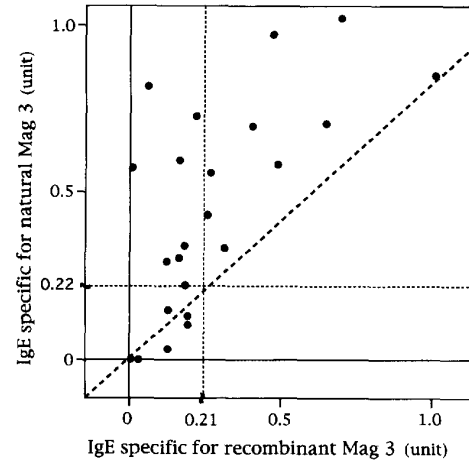


Fig. 4. Correlation between specific IgE levels to recombinant Mag 3 and those to natural Mag 3. Closed circles and thin broken lines indicate specific IgE levels of individual mite-allergic subjects ($n = 23$) and cut-off values, respectively.

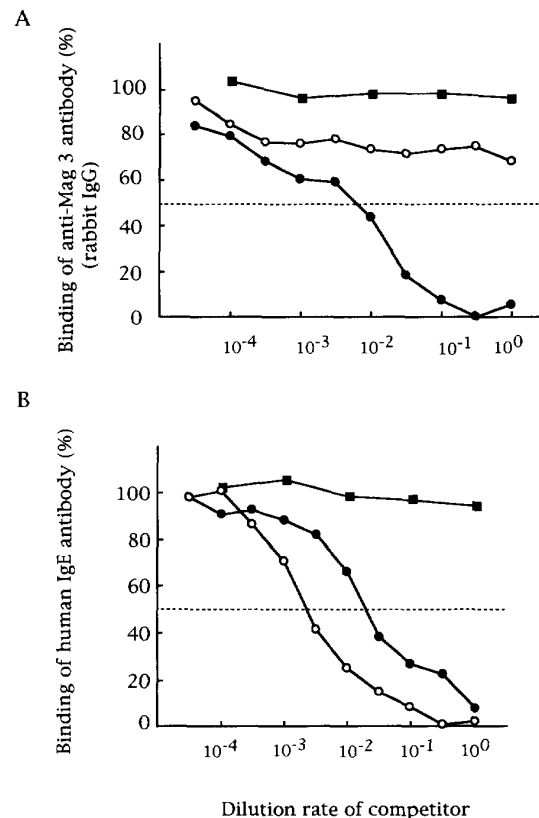


Fig. 5. Competitive inhibition of human IgE-binding (A) and rabbit anti-recombinant Mag 3-binding (B) to natural Mag 3 with recombinant Mag 3. Each well was coated with natural Mag 3 (10 pmol). Before dispensing human pooled sera (A) or rabbit anti-recombinant Mag 3 sera (B) to the antigen-coated wells, the corresponding sera were incubated with a series of properly diluted natural Mag 3 (●), recombinant Mag 3 (○), and cedar pollen extract (■) solutions at initial concentrations of 500 pmol, 500 pmol and 50 µg/ml, respectively. Inhibitory potencies of the recombinant Mag 3 were measured at 415 nm according to the standard ELISA method.

that natural Mag 3 is a new species of high-molecular-weight allergen.

Some mite allergens have been shown to be digestive enzymes. Allergens in groups 1 and 4 are cysteine protease and α -amylase, respectively, and allergens in groups 3 and 6 correspond to trypsin-like serine and chymotrypsin-like serine proteases, respectively. As might be predicted, these allergens are found both in extracts of mite bodies and feces (Chua *et al.*, 1988; Simpson *et al.*, 1989; Lake *et al.*, 1991a, 1991b; Stewart *et al.*, 1992; Yasueda *et al.*, 1986; Smith *et al.*, 1994). *Der p 1* has been localized to both the gut and fecal pellet by microscopic observation (Thomas *et al.*, 1991). Our preliminary experiments showed that natural Mag 3 was included in the mite body extract but not in the mite feces extract using anti-recombinant Mag 3 as a probe. When mites were examined directly by histochemical methodologies (Fig. 4), Mag 3 did not co-localize with those digestive enzymes but was found in the circumferential tissue, presumably interstitial tissue, of the esophagus, gut and the other internal organs in mites.

Major allergens have been defined so far in terms of both the frequency of the response by allergic subjects and proportion of total IgE directed against these allergens. However, the values obtained for the frequency of response and capacity of IgE-binding were largely dependent upon the selection of a group of patients (Tovey and Baldo, 1985, 1987a; Tovey *et al.*, 1987b,c; Baldo *et al.*, 1989; Wahl *et al.*, 1989; Nakanishi and Shimokata, 1990; Colloff *et al.*, 1992), especially in the case of random selection of bronchial asthmatic patients positive to skin test to mites (Aki *et al.*, 1994a, 1994c, 1995). We consider that these values of the interest allergens should be represented as relative values to the major allergen. The binding frequency and capacity of human IgE specific for natural Mag 3 were comparable to those to *Der f 2*. Consequently, we conclude that natural Mag 3 should be considered as an important allergen.

Specific IgE in pooled sera of patients sensitive to mites, which reacted with both recombinant Mag 3 and natural Mag 3, responded strongly to natural Mag 3 rather than recombinant Mag 3. The recombinant Mag 3 suppressed binding of human IgE specific for natural Mag 3 to only 30%. These results suggest that other epitopes should reside in natural Mag 3 molecules. On the other hand, recombinant Mag 3 inhibited the reaction of rabbit anti-recombinant Mag 3 to natural Mag 3 at 50 pmol. Up to now, we have no evidence sufficient to determine whether this phenomenon is due to steric hindrance of a shared epitope in the natural Mag 3 molecule or to a difference in avidity between analogous epitopes shared by natural Mag 3 and recombinant Mag 3.

The CNBr degradation of the natural Mag 3 resulted in two discrete fragments (30 and 45 kD). Ten amino acid sequences at the N-terminal of both fragments were identified, but these sequences were not found in the deduced amino acid sequence of cloned *mag 3*. We are presently investigating these questions.

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REFERENCES

- Abe T. and Ishii A. (1982) Purification of a murine IgE-inducing antigen extracted from the house dust mite, *Dermatophagoides pteronyssinus*. *Molec. Immun.* **19**, 339–346.
- Aki T., Ono K., Paik S. Y., Wada T., Jyo T., Shigeta S., Murooka Y. and Oka S. (1994a) Cloning and characterization of cDNA coding for a new allergen from the house dust mite, *Dermatophagoides farinae*. *Int. Arch. Allergy Immun.* **103**, 349–356.
- Aki T., Ono K., Hidaka Y., Shimonishi Y., Jyo T., Wada T., Yamashita M., Shigeta S., Murooka Y. and Oka S. (1994b) Structure of IgE epitopes on a new 39 kD allergen molecule from the house dust mite, *Dermatophagoides farinae*. *Int. Arch. Allergy Immun.* **103**, 357–364.
- Aki T., A. Fujikawa T., Wada T., Jyo S., Shigeta S., Murooka Y., Oka S. and Ono K. (1994c) Cloning and expression of cDNA coding for a new allergen from the house dust mite, *Dermatophagoides farinae*: homology with human heat shock cognate proteins in the heat shock protein 70 family. *J. Biochem.* **115**, 435–440.
- Aki T., Kodama T., Fujikawa A., Miura K., Shigeta S., Wada T., Jyo T., Murooka Y., Oka S. and Ono K. (1995) Immunohistochemical characterization of recombinant and natural tropomyosins as a new allergen from the house dust mite, *Dermatophagoides farinae*. *J. Allergy Clin. Immun.* **96**, 74–83.
- Arlian L. G., Bernstein I. L., Geis D. P., Vyszynski-Moher D. L., Gallagher J. S. and Martin B. (1987a) Investigations of culture medium-free house dust mites. III. Antigens and allergens of body and fecal extract of *Dermatophagoides farinae*. *J. Allergy Clin. Immun.* **79**, 457–466.
- Arlian L. G., Bernstein I. L., Vyszynski-Moher D. L. and Gallagher J. S. (1987b) Investigation of culture medium-free house dust mites. IV. Cross antigenicity and allergenicity between the house dust mites, *Dermatophagoides farinae* and *D. pteronyssinus*. *J. Allergy Clin. Immun.* **79**, 467–476.
- Arlian L. G. (1991) House-dust-mite allergens: a review. *Exp. appl. Acarol.* **10**, 167–186.
- Baldo B. A., Ford S. A. and Tovey E. R. (1989) Toward a definition of the "complete" spectrum and rank order of importance of the allergens from the house dust mite: *Dermatophagoides pteronyssinus*. *Adv. Biosci.* **74**, 13–31.
- Brody A. R., McGrath J. C. and Wharton G. W. (1972) *Dermatophagoides farinae*: the digestive system. *NY Entomol. Soc. LXXX*, 152–177.
- Chua K. Y., Stewart G. A., Thomas W. R., Simpson R. J., Dilworth R. J., Plozza T. M. and Turner K. J. (1988) Sequence analysis of cDNA coding for a major house dust mite allergen, *Der p 1*. Homology with cysteine protease. *J. exp. Med.* **167**, 175–182.
- Colloff M. J., Howe C. W., McSharry C. and Smith H. V. (1992) Characterization of IgE antibody binding profiles of sera from patients with atopic dermatitis to allergens of the domestic mites *Dermatophagoides pteronyssinus* and *Euroglyphus maynei*, using enhanced chemiluminescent immunoblotting. *Int. Arch. Allergy Immun.* **97**, 44–49.

- Frangioni J. V. and Neel B. G. (1993) Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Analyt. Biochem.* **210**, 179–187.
- Fujii S., Ono K., Shigeta S., Oka S. and Yamashita U. (1996) B cell mitogenic activity of house dust mite, *Dermatophagoides farinae*, antigen. *Immun. Lett.* (In press).
- King T. P., Hoffman D., Lowenstein H., Marsh D. G., Platts-Mills T. A. E. and Thomas W. (1994) Allergen nomenclature. *Int. Arch. Allergy Immun.* **105**, 224–233.
- Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lake F. R., Thompson P. J., Ward L. D., Simpson R. J. and Stewart G. A. (1991a) House dust mite derived amylase: allergenicity and physicochemical characterisation. *J. Allergy clin. Immun.* **87**, 1035–1042.
- Lake F. R., Ward L. D., Simpson R. J., Thompson P. J. and Stewart G. A. (1991b) Allergenicity and physicochemical characterisation of house dust mite derived amylase. *Int. Arch. Allergy appl. Immun.* **94**, 357–358.
- Nakanishi K. and Shimokata K. (1990) Immunoblot analysis of *Dermatophagoides farinae* antigen. *Ann. Allergy* **64**, 219–223.
- O'Neil G. M., Donovan G. R. and Baldo B. A. (1994) Cloning and characterization of a major allergen of the house dust mite, *Dermatophagoides pteronyssinus*, homologous with glutathione S transferase. *Biochim. Biophys. Acta* **1219**, 521–528.
- Platts-Mills T. A. E., Thomas W. R., Aalberse R. C., Vervloet D. and Chapman M. D. (1992) Dust mite allergens and asthma: report of a second international workshop. *J. Allergy clin. Immun.* **89**, 1046–1060.
- Sanger F., Nicklen S. and Coulson A. R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. natl. Acad. Sci. U.S.A.* **74**, 5463–5467.
- Shigeta S., Tanaka S., Yoshizane T., Yamaguchi T., Jyo T., Wada T., Oka S. and Ono K. (1995) High-molecular-size mite allergens with high carbohydrate content in mite extract containing mite feces of *Dermatophagoides farinae*. *Japan J. Allergol.* **44**, 170–181.
- Simpson R. J., Nice E. C., Moritz R. L. and Stewart G. A. (1989) Structural studies on the allergen *Der p 1* from the house dust mite *Dermatophagoides pteronyssinus*: similarity with cysteine proteases. *Prot. Seq. Data Anal.* **2**, 17–21.
- Smith D. B. and Johnson K. S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31–40.
- Smith W. A., Chua K. Y., Kuo M. C., Rogers B. L. and Thomas W. R. (1994) Cloning and sequencing of the *Dermatophagoides pteronyssinus* group III allergen, *Der p III*. *Clin. exp. Allergy* **24**, 220–228.
- Stewart G. A. and Turner K. J. (1980a) Physicochemical and immunochemical characterization of the allergens from the mite *Dermatophagoides pteronyssinus*. *Aust. J. exp. biol. med. Sci.* **58**, 259–274.
- Stewart G. A. and Turner K. J. (1980b) Physicochemical and immunochemical characterization of the high molecular weight allergens from *Dermatophagoides pteronyssinus* with particular reference to the Tridacnin, Con-A and S107 reactive components. *Aust. J. exp. biol. med. Sci.* **58**, 275–288.
- Stewart G. A., Butcher A. and Turner K. J. (1983) Characterization of a very high density lipoprotein allergen, Dpt 4, from the house dust mite *Dermatophagoides pteronyssinus*. *Int. Arch. Allergy appl. Immun.* **71**, 340–345.
- Stewart G. A., Ward L. D., Simpson R. J. and Thompson P. J. (1992) The group III allergen from the house dust mite *Dermatophagoides pteronyssinus* is a trypsin-like enzyme. *Immunology* **75**, 29–35.
- Thomas B., Heap P. and Carswell F. (1991) Ultrastructural localization of the allergen *Der p I* in the gut of the house dust mite *Dermatophagoides pteronyssinus*. *Int. Arch. Allergy appl. Immun.* **94**, 365–367.
- Tongu Y., Ishii A. and Oh H. (1986) Ultrastructure of house-dust mites, *Dermatophagoides farinae* and *D. pteronyssinus*. *Japan J. Sanit. Zool.* **37**, 237–244.
- Tovey E. R. and Baldo B. A. (1985) Detection of house dust mite allergens and frequency of IgE binding following electrophoresis and enzyme immunoassay. *Int. Arch. Allergy appl. Immun.* **76**, 82–85.
- Tovey E. R. and Baldo B. A. (1987a) Comparison by electrophoresis of IgE-binding components in extracts of house dust mite bodies and spent mite culture. *J. Allergy clin. Immun.* **79**, 93–102.
- Tovey E. R., Ford S. A. and Baldo B. A. (1987b) Protein blotting on nitrocellulose: some important aspects of the resolution and detection of antigens in complex extracts. *J. Biochem. Biophys. Meth.* **14**, 1–17.
- Tovey E. R. and Baldo B. A. (1987c) Characterisation of allergens by protein blotting. *Electrophoresis* **8**, 452–463.
- Voorhorst R., Spijkema F. T. M., Varekamp M. J., Leupen M. J. and Lyklema A. W. (1967) The house dust mite (*Dermatophagoides pteronyssinus*) and the allergens it produces. Identity with the house dust mite allergen. *J. Allergy* **39**, 325–339.
- Wahl R., Meineke D., Maasch H. J. and Schultze-Werninghaus G. (1989) Investigation of the allergenic activity of isolated fractions of a standardized partly purified whole mite body (*Dermatophagoides pteronyssinus*) extract. *Int. Arch. Allergy appl. Immun.* **90**, 224–229.
- Yamaguchi T., Ono K., Shigeta S., Wada T., Ohtsuka T., Jyo T. and Oka S. (1993) Differences in response of patient's blood cells with bronchial asthma to diverse house dust mite allergens by histamine release assay. *Japan J. Allergol.* **42**, 1754–1763.
- Yamaguchi T., Tanaka S., Shigeta S., Wada T., Tsuboi S., Ohtsuka T., Katsutani T., Jyo T., Oka S. and Ono K. (1994a) Low-molecular-size allergens, LM-1s, in feces extract of *Dermatophagoides farinae* which elicit histamine release from washed blood cells of patients with bronchial asthma. *Japan J. Allergol.* **43**, 701–710.
- Yamaguchi T., Tanaka S., Shigeta S., Wada T., Tsuboi S., Ohtsuka T., Katsutani T., Jyo T., Oka S. and Ono K. (1994b) A low-molecular-size LM2 allergen in mite, *Dermatophagoides farinae*, extracts containing feces which elicits conjunctival reactions in patients with bronchial asthma. *Japan J. Allergol.* **43**, 815–824.
- Yasueda H., Mita H., Yui Y. and Shida T. (1986) Isolation and characterization of two allergens from *Dermatophagoides farinae*. *Int. Arch. Allergy appl. Immun.* **81**, 214–223.