

Measurement of IgG subclass antibodies to the group II antigen of *Dermatophagoides pteronyssinus* (Der p II) in sera from children with bronchial asthma

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Seven IgG-binding components in crude mite allergen extracted from *Dermatophagoides pteronyssinus* (Der p) were identified by immunoblotting analysis. Of these components, the 15-kDa protein was strongly recognized by IgG from patients with bronchial asthma, whereas it was less recognized by IgG from healthy individuals. Then, the 15-kDa protein was purified by reverse-phase high performance liquid chromatography and the protein was demonstrated to induce immediate positive skin reactions in patients with bronchial asthma. The N-terminal amino acid sequence of the 15-kDa protein was also determined and was in agreement with that of Der p II. In an attempt to understand the role of IgG subclass antibody of patients with bronchial asthma, we quantitated the antibodies of IgG subclass to Der p II. The levels of antibodies of total IgG and of all IgG subclasses in patients with bronchial asthma who had never received immunotherapy were higher than those of nonatopic healthy individuals. The levels of Der p II-specific IgG1 and IgG4 in asthmatic patients were 1.97 times and 2.20 times higher, respectively, than those in the control group. The present study demonstrates that patients with bronchial asthma are able to produce antibodies of all IgG subclasses specific to Der p II in response to natural exposure to mite antigen.

INTRODUCTION

The house dust mite is recognized as a major causative allergen for atopic bronchial asthma and allergic rhinitis. Two species of mites, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, are well known as the major allergens. Many IgE-binding components in *Dermatophagoides* species have been identified by crossed radioimmuno-electrophoresis and immunoblotting.¹⁻³ Group I and group II antigens with apparent molecular masses of 24 kDa and 15 kDa, respectively, have been purified.^{4,5}

More than 80% of mite-allergic patients have measurable IgE antibodies to these allergens.⁶

The immune response to mite antigens has been studied. Chapman et al⁷⁻⁹ reported that there is a good quantitative correlation between the levels of IgG and IgE antibody, which suggests that these two isotypes are under common genetic and immunoregulatory control. In a controlled study of immunotherapy carried out using aqueous *Dermatophagoides pteronyssinus* (Der p) extract, a significant increase of anti-Der p I IgG antibody, accompanied by a changeless or slightly decreased concentration of IgE antibody, has been reported.^{8,10} The significance, however, of the appearance of IgG antibody is not clear. IgG antibody may act as a blocking antibody after immunotherapy.

In the present study, we focused on the IgG-binding components in mite allergens and purified group II

antigen from *Dermatophagoides pteronyssinus* (Der p II), which was significantly recognized by IgG antibodies from patients with bronchial asthma. We examined the correlation between the appearance of IgG antibody and IgE RAST score, and the levels of IgG subclass antibodies to purified Der p II in sera from patients with atopic bronchial asthma.

MATERIALS AND METHODS

Subjects

Sera from 40 patients with atopic bronchial asthma (aged 1-15 years old, mean 7.1 years old), who had never received immunotherapy, and sera from 20 nonatopic individuals (ten children, aged 2-15 years old, mean 8.6 years old, and ten adults) as controls were studied. Thirty-three patients exhibited positive radioallergosorbent tests (RAST score $\geq 3+$) (Pharmacia LKB Biotechnology AB Uppsala, Sweden).

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Preparation of Crude Mite Extract
Dermatophagoides pteronyssinus (*Der p*), which was a generous gift of Dr. Takahashi (Institute of Public Health of Hokkaido), was cultivated in a flat type flask containing dried fish powder and dried yeast (Wako Pure Chemical Industries, Ltd. Osaka) at 25 °C in 75% humidity for 2 months according to the method of Miyamoto et al.¹¹ After cultivation, culture media and mite bodies were collected by repipetting flotation on saturated NaCl solution as described by Sasa et al.¹² Collected mite bodies were then homogenized with a polytron homogenizer. After homogenization, the extract was centrifuged at 100,000 g for 20 minutes. The supernatant was then dialyzed against 10 mM Tris-HCl buffer (pH 7.4) for two days. The protein content was determined by the method of Lowry et al.¹³ and stored at -80 °C.

Identification of IgG-Binding Components in Crude Mite Extract from Dermatophagoides pteronyssinus by Western Blotting
Crude mite extract (3-μg protein/lane) was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli¹⁴ and separated proteins were electrotransferred to a nitrocellulose sheet by the method of Towbin et al.¹⁵ at 40 V overnight. The nitrocellulose sheet was incubated with 5% (wt/vol) skim milk containing 0.2% (vol/vol) Triton X-100 in 10 mM phosphate buffered saline, pH 7.4 (PBS) at room temperature for two hours to block non-specific binding. The sheet was then incubated with sera [1:50 in 10 mM PBS containing 0.2% (vol/vol) Triton X-100 and 5% (wt/vol) skim milk] overnight at 4 °C, and washed with 0.2% (vol/vol) Triton X-100 in PBS for 60 minutes with three changes. Next, the sheet was incubated with horseradish peroxidase-conjugated goat anti-human IgG (TAGO, Inc, Burlingame, CA) [1:500 in PBS containing 0.2% (vol/vol) Triton X-100 and 5% (wt/vol)

skim milk] at 37 °C for 90 minutes. After washing with 0.2% (vol/vol) Triton X-100 in PBS for 60 minutes followed by another washing with PBS for 20 minutes, the sheet was substrated with diaminobenzidine tetrahydrochloride (DAB) (Katayama Chemical Co. Ltd., Tokyo).

Purification of the 15-kDa Protein and Analysis of N-Terminal Amino Acid Sequence

The crude mite extract was applied to a Sephadex G 75 column and eluted with 10 mM Tris buffer (pH 7.4). The fractions were then analyzed by SDS-PAGE. The fractions containing the protein with a molecular mass of 15 kDa were collected and applied to reverse-phase high performance liquid chromatography (HPLC) using a C8 column (Cosmosil, Nakarai Industrial Co. Ltd., Tokyo) equilibrated with 0.1% (vol/vol) trifluoroacetic acid (TFA). Bound materials were eluted with a linear gradient of 20% to 80% (vol/vol) acetonitrile in 0.1% TFA at a flow rate of 0.5 mL/min. The effluent was monitored at 214 nm, and the fractions obtained were analyzed by SDS-PAGE. N-terminal amino acid sequencing of the 15-kDa protein (approximately 10 nmol) was performed with an amino acid sequencer (470A: Applied Biosystems, Inc., Foster City, CA) and an on-line 120A HPLC.

Skin Testing

The purified *Der p* II was dialyzed against 10 mM Tris-buffer (pH 7.4) and passed through a 0.45-μm filter unit (Millipore, Bedford, USA). The antigen was diluted to a final protein concentration of 4×10^{-4} μg/mL in buffered saline and 0.02 mL was injected intradermally into the upper arm of the test subjects. Test sites were examined 15 minutes after injection. The skin tests were performed on ten patients with bronchial asthma (aged 3-14 years old) who had positive IgE RAST scores and nonatopic individuals (five healthy children and five healthy adults) as controls.

Quantitation of IgG Subclasses to Der p II by ELISA

Mouse monoclonal antibodies to human IgG1 (HP6069), IgG2 (HP6014), IgG3 (HP6050), and IgG4 (HP6011) were kindly donated by the World Health Organization (WHO). In the present study, anti-human IgG1 was diluted 1:400 and IgG2, IgG3, and IgG4 were diluted 1:200 in PBS containing 0.01% (vol/vol) Triton X-100 (TX/PBS) before use. Purified *Der p* II diluted at 10 μg/mL in 0.1 M NaHCO₃, pH 8.4 (50 μL/well) was added to the microtiter wells (Dynatech Laboratories Inc.) and incubated at 4 °C overnight. The plates were washed three times with PBS and supplemented with 200 μL of 0.25% gelatine in 10 mM PBS containing 0.01% (vol/vol) Triton X-100 (GL/TX/PBS) to block the nonspecific binding. After blocking for two hours at room temperature, the plates were washed three times with TX/PBS, and then sample sera or pooled sera from ten healthy individuals diluted 1:50 and were added to each well. Following a 2-hour incubation at 37 °C and three washings with TX/PBS, the monoclonal anti-human IgG or IgG1-4 described above was added and incubated at 37 °C for two hours. After washing, peroxidase-conjugated goat anti-mouse IgG (TAGO, Inc, Burlingame, CA) was added (1/1000 dilution) and further incubated at 37 °C for two hours. Finally, 100 μL of *o*-phenylenediamine (Kanto Chemical Inc., 1 mg/mL) in 0.15 M citrate-phosphate buffer (pH 4.6) containing H₂O₂ (0.001%) was added as substrate and incubated for 20 minutes. After stopping the reaction with 2 M H₂SO₄, absorbance at 492 nm was read in a multiplate reader. Absorbances of sample sera were compared with those of pooled sera from healthy individuals. The relative index was determined by dividing the absorbance of the sample serum by that of the pooled sera from healthy individuals.

RESULTS

Identification of IgG-Binding Components in Crude Mite Extract from *Dermatophagoides pteronyssinus* (Der p) by Immunoblotting Analysis

IgG from patients with bronchial asthma appeared to recognize seven proteins with reduced denatured molecular masses of 95, 88, 70, 65, 53, 32, and 15 kDa. (Fig 1, lanes 3 to 10). Of these components, the 15-kDa protein band was detected by sera from 29 (72.5%) of the patients with bronchial asthma, whereas this protein was detected by only four (20%) of the control sera (Fig 1, lane

1 and 2). Table 1 summarizes the results of immunoblotting analysis of IgG-binding components in mite antigens for sera from 40 patients with bronchial asthma and from 20 controls. These data indicate that only the 15-kDa protein made a difference between the patients and controls when IgG-binding in sera was examined by immunoblotting analysis.

Correlation Between the Appearance of the IgG Antibody to the 15-kDa Protein and IgE RAST Score

When the IgE RAST score and the appearance of IgG antibody to the

15-kDa protein in patients with bronchial asthma were compared, the % of the positivity of IgG antibody to the 15-kDa protein increased with the IgE RAST score (Table 2). Twenty-seven (81%) of 33 patients with bronchial asthma had IgE RAST score over 3+ and they exhibited an IgG antibody response to the 15-kDa protein. Two (28%) of seven patients, in whom IgE RAST scores were negative, showed IgG antibody responses. These data indicate that there was a good correlation between the degree of IgE RAST score and the appearance of IgG antibody to the 15-kDa protein.

Purification and Analysis of the 15-kDa Protein

The mite antigen purified partially by gel filtration chromatography was further purified by reverse-phase HPLC using a C8 column (Fig 2, panel A). The first 20 residues of the amino-terminal in the amino acid sequence of this protein had been determined. The sequences obtained by the present study were consistent with those obtained from cDNA of *Der p II* recently reported by Chua et al.¹⁶

The purified antigen was analyzed by immunoblotting with human sera. *Der p II* was detected only by pooled sera from bronchial asthma patients, whereas no band was detected in sera from healthy controls (Fig 2, panel B).

Next, we examined whether the purified *Der p II* possesses biologic

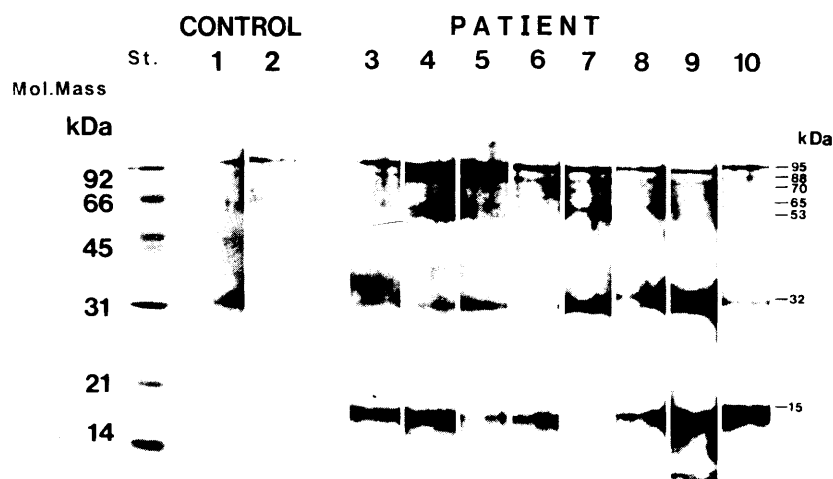


Figure 1. Immunoblotting analysis of crude extract from *Dermatophagoides pteronyssinus* with sera from allergic and nonallergic individuals. Crude extract from *Der p* was electrophoresed on 15% polyacrylamide gel, transferred to a nitrocellulose sheet, and immunostained with sera from controls (lanes 1 and 2), or sera from patients with bronchial asthma (lanes 3 to 10) as described under "Materials and Methods."

Table 1. IgG-Binding Components in Patients with Bronchial Asthma and Normal Controls*

Subject		IgG-Binding Components (kDa)†						
		95	88	70	65	53	32	15
Normal controls	Positive staining number	10	17	17	17	15	16	4
	%	50.0	85.0	85.0	85.0	75.0	80.0	20.0
Patients with asthma	Positive staining number	31	37	37	37	35	31	29
	%	77.5	92.5	92.5	92.5	87.5	77.5	72.5

* Crude extract from *Der p* was analyzed by immunoblotting using sera from control (n = 20) or patients with asthma (n = 40) as described under "Materials and Methods."

† For the seven protein bands detected, the number of samples that exhibited positive stainings for each band were counted and the % of positive stainings are expressed.

Table 2. Correlation Between IgE RAST Score and IgG Antibody to the 15-kDa Protein

RAST Score*	Total Number	Positive	
		Number	%
1+	4	1	25.0
2+	3	1	33.0
3+	4	4	100
4+	29	23	79.3
total	40	29	72.5

* The IgE RAST score and the appearance of IgG antibody to the 15-kDa protein in asthmatic patients were compared.

activity. Ten normal children and ten patients with bronchial asthma underwent skin testing using the purified protein. All of the patients with bronchial asthma exhibited immediate skin reactions, whereas the protein caused no positive skin reactions in any of the nonallergic children. This result demonstrates that the *Der p* II we purified clearly induced the immediate skin reactions in mite-allergic patients.

Determination of IgG and IgG Subclasses to *Der p* II by ELISA

Titers of IgG or IgG subclasses to the purified *Der p* II in sera from healthy individuals and individuals with bronchial asthma, who had never received immunotherapy, were determined by ELISA in comparison to pooled normal sera. The levels of total IgG and of all IgG

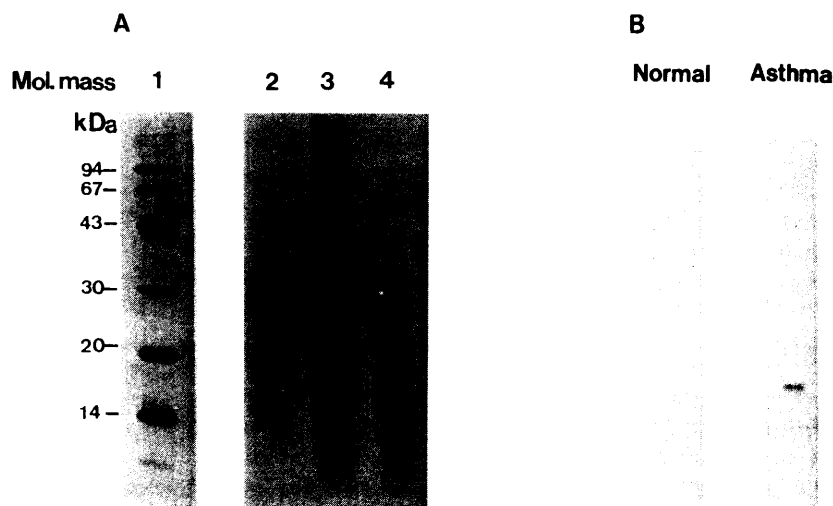
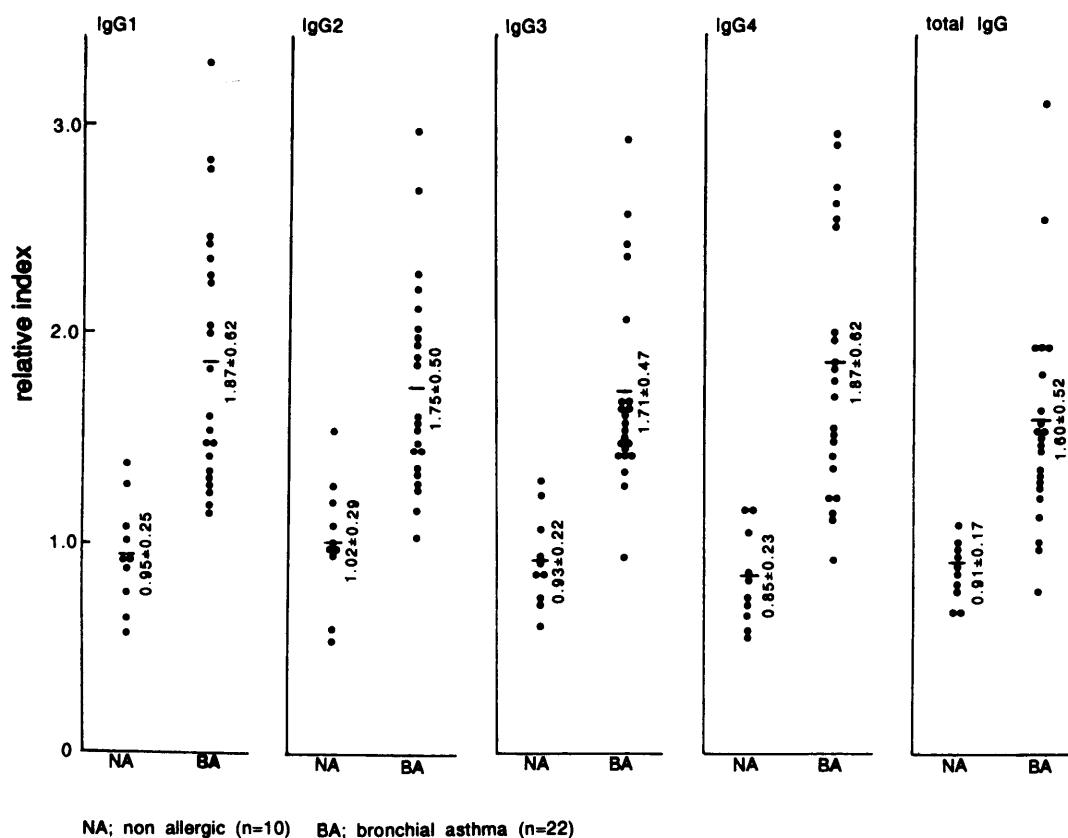


Figure 2. Electrophoretic and immunoblotting analysis of *Der p* II. Crude mite extract from *Dermatophagoides pteronyssinus* (panel A, lane 3), partially purified proteins by gel filtration chromatography over Sephadex G 75 (panel A, lane 4) and *Der p* II purified by reverse-phase HPLC (panel A, lane 2) were analyzed by SDS-PAGE. The molecular weight standard (panel A, lane 1) was also electrophoresed. The purified *Der p* II was then analyzed by immunoblotting with pooled sera from bronchial asthma patients or healthy controls (panel B).



NA; non allergic (n=10) BA; bronchial asthma (n=22)

Figure 3. Antibody of total IgG and IgG subclasses to *Der p* II in nonallergic and asthmatic children. The purified *Der p* II was adsorbed onto microtiter wells and the total IgG and IgG subclasses were quantitated as described under "Materials and Methods." The data express the relative index determined by dividing the absorbance of the sample sera from nonallergic (NA) children or from children with bronchial asthma (BA) by that of pooled sera from healthy individuals.

subclasses in patients with bronchial asthma were higher than those in nonatopic individuals (Fig 3). The increases of IgG subclasses specific to *Der p* II in patients with bronchial asthma appears to be especially prominent in IgG1 and IgG4 (1.97-fold and 2.20-fold, respectively) when compared with those in healthy individuals. The data presented demonstrate that patients with bronchial asthma are able to produce antibodies of all IgG subclasses specific to *Der p* II in response to natural exposure to mite antigen.

DISCUSSION

The house dust mite is well known as a major allergen responsible for atopic bronchial asthma in children. The *Der* I (molecular weight 24,000) and *Der* II (molecular weight 15,000) allergens are generally recognized as the major allergens, since immediate hypersensitivity to these allergens has been demonstrated in most mite-allergic patients by skin testing, histamine release, and measurements of serum IgE antibody.^{4,5,17}

In the present study, we identified IgG-binding components in crude mite antigen by immunoblotting analysis. IgG from patients with bronchial asthma appeared to recognize mainly seven proteins. Of these components, the 15-kDa protein, *Der p* II, was strongly detected by sera from 72.5% of the patients with bronchial asthma and 81% of the patients with positive RAST score, whereas this protein was hardly detected by control sera (20%).

There are some reports on IgG-binding components in mite antigens. Nakanishi et al¹⁸ reported, concerning the components of *Dermatophagoides farinae*, that a total of 27 IgG-binding components were identified and distributed over molecular range of 13 to 140 kDa. When responses of IgG4 antibody to mite antigens were analyzed by immunoblotting, the binding of specific IgG4 antibodies to mite anti-

gens was found in seven out of 30 sera tested. IgG4-binding components were proteins with apparent molecular masses of 13, 25, 43, and 50 kDa, which exhibited relatively low frequencies of binding of IgE antibodies.

Lin et al¹⁹ also examined IgE-binding and IgG-binding in crude extracts of *Der p* by immunoblotting technique. IgE bound mainly to proteins with apparent molecular masses of 14, 16, 27, 33, 43, 55, 60, and 110 kDa. IgG-binding components were similar to those that bound IgE, except that the 27-kDa band detected by IgG appeared to be fainter than that by IgE. The IgG4-reacting pattern was also similar to the IgE-reacting pattern in high IgE-sera, except for the 27-kDa protein.

In the present study the 27-kDa protein, probably *Der p* I, was hardly detected as well. It is likely that denaturation of *Der p* I by treatment of crude mite extract with heat and detergent for electrophoresis may reduce IgG binding to *Der p* I since treatment of *Der p* I with heat or detergent dramatically reduced the binding of monoclonal antibodies or IgE to *Der p* I in contrast to the heat stable property of *Der p* II.²⁰

Kemeny et al²¹ studied the subclasses of IgG to house dust mite by ELISA. IgG1 antibody to crude mite antigens was detected in most atopics (94%) and nonatopics (97%), and IgG4 antibody was detected to a higher degree in atopics (66%) than nonatopics (53%). While the concentrations of IgG1 antibodies were not significantly different between those two groups, those of IgG4 antibodies were lower in nonatopics.

Soliman et al²² also measured the concentration of mite-specific IgG using a double antibody ELISA. Sixty-three percent of asthmatic subjects exhibited positive IgG titers, whereas only 10% of control subjects were positive. When mite-specific IgG4 concentration was measured, only 10% of IgG-positive

asthmatic subjects had high titers of mite-specific IgG4. But all seven patients that had received mite antigen immunotherapy exhibited high titers of mite-specific IgG4.

Tsai et al²³ determined IgG4 antibody to mite antigens by ELISA using crude mite extracts. The titer of mite-specific IgG4 in the asthmatic patients was 2.23-times higher than that in the control group, and further increased 1.52-fold after receiving immunotherapy.

Chapman and Platts-Mills⁷ measured IgG antibodies to *Der p* using a partially purified fraction of mite extract containing the proteins with apparent molecular masses of 15 to 25 kDa. IgG antibody to this preparation was detected in sera from 94% of mite-allergic patients and from 30% of nonallergic control.

We focussed on *Der p* II since it makes a difference on IgG binding of control and patient groups. We quantitated IgG subclass antibodies to purified *Der p* II to understand the role of IgG antibodies of patients with bronchial asthma. Although the data presented in Figure 3 express the relative levels of IgG to *Der p* II in each of IgG subclass antibody, it is possible to conclude that all of the IgG subclass antibodies to this protein clearly increased in sera from patients with bronchial asthma when compared with healthy controls.

The present study suggests that all IgG subclasses of *Der p* II-specific antibody appear in sera of patients with bronchial asthma in response to natural exposure to *Der p* II since the children examined had never received immunotherapy.

The present study and other studies^{19,20} using immunoblotting analysis, however, have revealed that IgG antibody from allergic patients bound many components of mites. When IgG antibodies to mite antigens were measured by ELISA using crude preparations, the component of the antigens to which the level of IgG had elevated in patients' sera was undiscernible. The use,

therefore of purified antigen for ELISA is meaningful to measure the level of antibody specific to the antigen.

We have reported for the first time the levels of IgG subclasses of *Der p* II-specific antibodies in sera from children with bronchial asthma compared with healthy individuals. IgG subclasses to the other mite antigen should be measured using purified protein as well as purified *Der p* II.

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