

Differences in titres of IgE, IgG4 and other IgG subclass anti-Der p 2 antibodies in allergic and non-allergic patients measured with recombinant allergen

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Summary

Background The mite allergens are recognized as major causes of allergic disease such as bronchial asthma, allergic rhinitis and atopic dermatitis. The functions of allergen-specific IgG subclass antibodies are not defined.

Objective In order to clarify the relationship between IgE and IgG subclasses, we examined serum levels of the *Dermatophagoides pteronyssinus* group 2 (Der p 2)-specific antibodies of IgE, IgG total and IgG subclasses in children with mite allergy.

Methods We prepared a recombinant Der p 2 fusion protein and examined serum levels of Der p 2 antigen-specific antibodies by enzyme-linked immunosorbent assay (ELISA) systems developed in our laboratory using a recombinant Der p 2 as target antigen. Sera from 240 children with mite allergy and 25 controls were measured.

Results The serum levels of specific IgE and, to lesser degree, IgG4 were higher in allergic children than non-allergic controls, while in the levels of the other IgG subclasses there was no difference between the two groups. There was no correlation between levels of specific IgE and IgG4 or in those between specific IgG4 and other IgG subclasses.

Conclusion Results indicate that the induction of Der p 2-specific IgG4 in allergic diseases is independent to IgE as well as other IgG subclasses.

Keywords: *Dermatophagoides pteronyssinus* group 2 (Der p 2), ELISA, IgG subclass, recombinant allergen, IgE, IgG4

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Introduction

Allergens from *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f) are recognized as major causes of allergic disease such as bronchial asthma, allergic rhinitis and atopic dermatitis [1–3]. Major allergens in each species have been classified into three groups: group 1 (Der p 1, Der f 1), group 2 (Der p 2, Der f 2) and group 3 (Der p 3, Der f 3). Group 1 and group 2 antigens of both species have been purified and sequenced [4–8]. Der p 2 consists of 129 amino acid residues with six cysteines and has a calculated molecular

weight of 14 kDa. Der p 2 and Der f 2 have similar amino acid compositions [9,10], and more than 80% of mite-allergic patients have specific IgE antibody to the group 2 allergens [11].

It is reported that allergen-specific IgG total antibody is present in serum of allergic patients, although the exact function is not defined. IgG subclass antibodies have been examined for inhalant allergens [12,13], mite [14,15], foods [13,15–17], venoms [18], fungus [19,20] and others [21,22]. The increase of allergen-specific IgG4 antibodies after immunotherapy has been shown and these IgG4 antibodies are thought to act as blocking antibody [15,23]. Antibody responses of other IgG subclasses against allergens were inconsistent [14,18–21,24,25]. These forced us to study antibody responses

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of IgE and IgG subclasses to purified major allergen of *Dermatophagoides pteronyssinus*, the Der p 2. The purification of Der p 2 from mite extracts is quite complicated [5,6,8] and preparation of recombinant allergens will furnish numerous and stable allergens.

In the present study, we prepared a recombinant Der p 2 fusion protein, developed an enzyme-linked immunosorbent assay (ELISA) system with the recombinant protein and examined levels of the Der p 2-specific antibodies of IgE, IgG total and IgG subclasses in sera from children with mite allergy.

Materials and methods

Subjects

Sera were collected from 240 children aged from 17 months to 17 years who were diagnosed by allergologists at our hospital and other affiliated hospitals as having bronchial asthma, allergic rhinitis and atopic dermatitis. All of them exhibited positive IgE CAP RAST to Der p (score > 3, Pharmacia Biotechnology, Uppsala, Sweden) and have not received immunotherapy. Control sera were collected from 25 children with ages ranging from 10 months to 14 years, who had no atopic history or symptoms of allergic diseases.

Production and purification of recombinant Der p 2 fusion protein

cDNA encoding mature Der p 2 was amplified by the polymerase chain reaction (PCR). The PCR product was digested with EcoRI and PstI, and inserted into the maltose-binding protein fusion expression vector pMALc2 (New England Biolabs Inc, Beverly, MA, USA). *E. coli*, TB1, was transformed with the resulting construct and cultured in LB medium supplemented with 100 mg/mL of ABPC and 2% glucose. After Isopropyl- β -D-thiogalactopyranoside (IPTG, Sigma, St Louis, MO, USA) induction, the recombinant strain expressed a 56 kDa protein, that was a fusion protein consisting of maltose-binding protein (MBP, 42 kDa) and a peptide of Der p 2 (14 kDa). The recombinant Der p 2 fusion protein (r-Der p 2) was solubilized by sonication and purified by maltose affinity chromatography following gel filtration (Sephacryl S-200, Pharmacia) and ion-exchange chromatography on Mono Q (Pharmacia). The purified fusion protein was analysed by SDS-PAGE of polyacrylamide gradient from 5 to 20%.

Enzyme-linked immunosorbent assay

The plastic microplates (Costar 3590, Cambridge, MA, USA) were coated with r-Der p 2 fusion protein (1 mg/

well) (diluted in carbonate-buffer, pH 9.6) at room temperature for 2 h, following standing at 4°C for 16 h. The coated plates were washed three times with phosphate-buffered saline (PBS), pH 7.0, and were then incubated with 0.25 mL/well of a 1% bovine serum albumin-PBS solution at 4°C for 16 h to block any residual binding sites on the plates and stored at 4°C until use.

The following reactions were carried out at room temperature. One-tenth millilitres of serum samples, appropriately diluted with PBS 0.1% BSA, 0.1% Tween 20 and 0.01% thimerosal (PBS-dilution buffer) were added into a well in duplicate and incubated for 2 h. Serum dilutions were 1:400 for IgE detection, 1:500 for IgG total, 1:200 for IgG1, IgG2 and IgG4 subclasses and 1:25 for IgG3 subclass, respectively. For IgE antibody detection, a biotin-conjugated goat anti-human IgE antibody (OEM Concepts Inc., Toms River, NJ, USA), diluted 1:2000, was incubated for 2 h. Streptavidin conjugated peroxidase (Nichirei Co, Tokyo, Japan) was added to the washed plate, and incubated for 2 h. For the IgG total detection, a polyclonal peroxidase-conjugated goat antihuman IgG antibody (Tago, Inc, Burlingame, CA, USA) at 1:10 000 dilution was incubated in the same condition. For IgG subclasses' detection, mouse monoclonal antibodies to human IgG subclasses (Oxoid, Basingstoke, UK) were used each at 1:1000 dilution and incubated in the same condition. A peroxidase-conjugated goat polyclonal anti-mouse IgG (Tago) at 1:5000 dilution for IgG1 and 1:2000 dilution for IgG2, IgG3 and IgG4 subclasses were incubated. Colour was developed by addition of 0.1 mL of substrate solution containing 3 mg/mL of O-phenylenediamine/mL and 0.03% of H₂O₂ in 0.1 M citrate buffer (pH 6.0). The coloration was stopped by 0.1 mL/well of 2N HCl and absorbance at 492 nm was measured by a microplate reader, MTP-100 (Corona Electric, Katsuura, Japan). Values were expressed as arbitrary units (U) deduced from absorbances (492 nm) of a control serum (boy, 16 years, atopic dermatitis) that was standardized as having IgE: 1400 U, IgG total: 1150 U, IgG1: 1000 U, IgG2: 1000 U, IgG3: 700 U, IgG4: 900 U.

Reproducibility of ELISA

Intra-assay coefficient of variations (CV) of each system was determined by assaying (in duplicate) three samples with low, middle and high values, each 20 times in the same assay and inter-assay coefficient of variations were measured for the three samples, each five time in five assays done at different times.

Statistical analysis

Data between the allergic group and the control group were examined by Student's *t*-test.

Results

Purity of Der p 2 fusion protein

As shown in Fig. 1, the purified Der p 2 fusion protein showed a single band at an expected molecular weight of 56 kDa (14 kDa for Der p 2 + 42 kDa for MBP).

The reliability of ELISA for antibody to Der p 2

The reliability of ELISA systems determining levels of the Der p 2-specific antibodies of IgE, IgG total and IgG

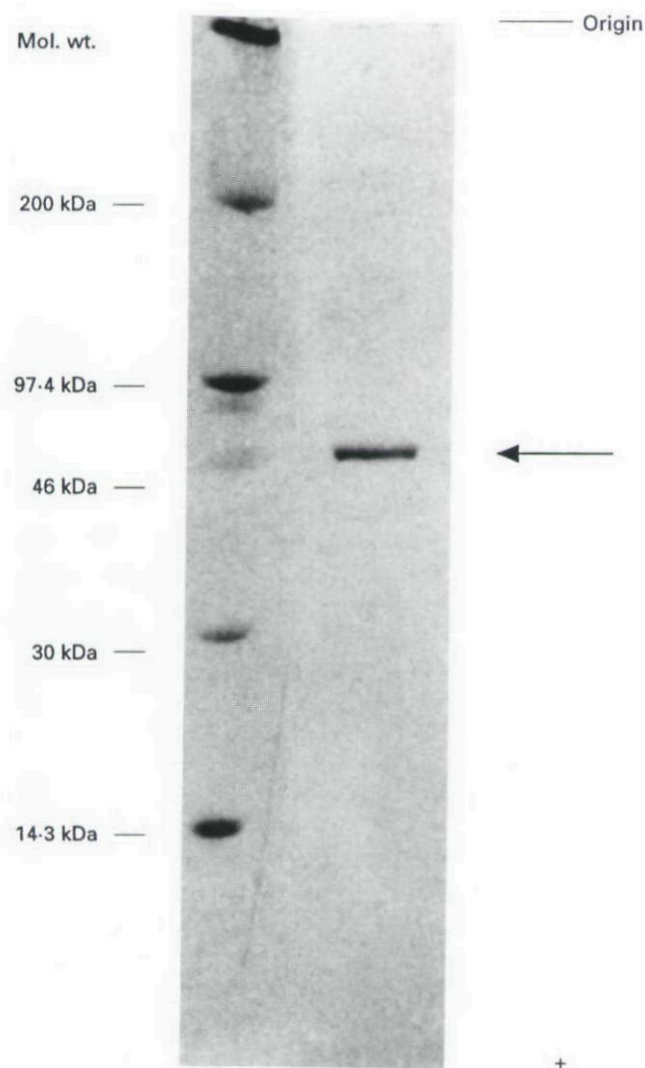


Fig. 1. SDS-PAGE gradient gel (5–20%) of purified recombinant Der p 2 fusion protein. The band in the right lane indicated by arrow represents purified recombinant Der p 2 fusion protein. The bands in the left lane represents marker proteins.

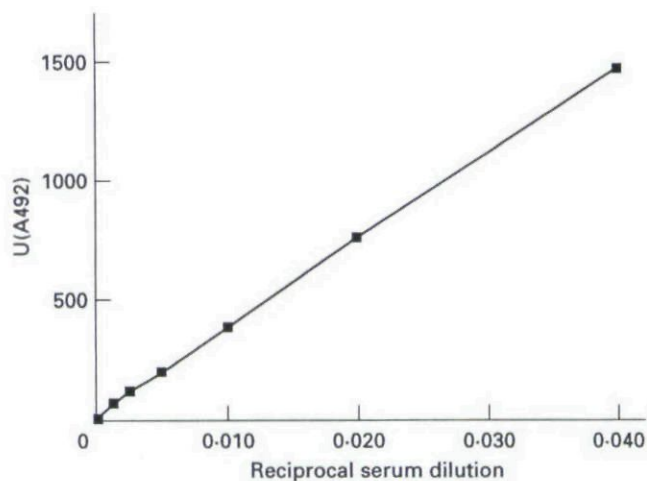


Fig. 2. A regression curve of Der p 2 specific IgG4 antibody by ELISA.

subclasses were assessed using a patient's serum with high total serum IgE (16 000 U/L) and IgE CAP RAST to Der p (over 100 U/mL). Every ELISA system gave a linear regression curve against the reciprocal dilution of the serum. A typical regression curve obtained for IgG4 subclass antibody is represented in Fig. 2. A possible non-specific binding of immunoglobulins to Der p 2 in the ELISA system was ruled out by the following experiments: individual patients' sera ($n = 13$) with different amount of serum IgG levels (from 900 mg/dL to 1900 mg/dL) were determined for their levels of the Der p 2-specific IgG total and a correlation study was made between the values of serum IgG and the Der p 2-specific IgG total (not shown). There was a poor correlation between the two ($r^2 = 0.128$); the effect of purified soluble Der p 2-fusion protein on the binding of immunoglobulin was assessed in the ELISA for Der p 2-specific IgG total. The binding of IgG total was clearly decreased in accord with the amount of purified soluble Der p 2-fusion protein added in the reaction.

Table 1 summarizes the CVs of intra-and inter-assay of ELISA for IgG total, IgG subclasses and IgE.

Comparisons of antibody level between allergic children and non-allergic controls

The levels (mean $\pm$ SD) of specific antibodies of IgE, IgG total and IgG subclasses against recombinant Der p 2 were determined for two groups, one allergic children and the other non-allergic controls (Table 2). Significant statistical differences between the two groups were observed in the levels of specific IgE and IgG4, but not in others (IgE: $P = 0.0008$; IgG4: $P = 0.0033$).

Table 1. Intra- and inter-assay of coefficient of variations in each ELISA

		Low	Middle	High
IgE	Levels (U)*	482.7 ± 34.7 SD	721.9 ± 57.5 SD	1057 ± 74.4 SD
	Intra, CV%	4.1	3.7	5.8
	Inter, CV%	9.9	10.7	4.9
IgG total	Levels (U)	175.5 ± 20.7 SD	389.0 ± 40.0 SD	8141.1 ± 66.7 SD
	Intra, CV%	13.5	8.4	8.5
	Inter, CV%	15.7	6.1	6.7
IgG1	Levels (U)	287.4 ± 6.7 SD	543.0 ± 10.0 SD	1343.6 ± 61.9 SD
	Intra, CV%	10.1	7.9	4.6
	Inter, CV%	13.7	6.3	3.9
IgG2	Levels (U)	392.5 ± 32.7 SD	650.0 ± 43.2 SD	1096.9 ± 59.9 SD
	Intra, CV%	8.3	6.7	5.5
	Inter, CV%	12.6	11.9	7.5
IgG3	Levels (U)	114.7 ± 16.0 SD	284.3 ± 31.9 SD	535.6 ± 40.4 SD
	Intra, CV%	13.9	11.2	7.5
	Inter, CV%	10.1	9.6	7.8
IgG4	Levels (U)	165.9 ± 21.3 SD	368.4 ± 39.7 SD	694.4 ± 68.4 SD
	Intra, CV%	12.8	10.7	9.8
	Inter, CV%	13.9	10.9	7.9

* Mean ± SD deduced from intra-assay.

Correlation study among IgE, IgGtotal and IgG subclasses

In allergic children, there was a significant correlation ($r^2 = 0.445$) between total serum IgE and the specific IgE level, against recombinant Der p 2 (Fig. 3a). Concerning the specific antibody of IgG subclasses, positive correlations were observed between levels of IgGtotal and IgG1 ($r^2 = 0.466$), IgG2 ($r^2 = 0.217$) and IgG3 ($r^2 = 0.356$),

but not of IgG4 ($r^2 = 0.057$) (Fig. 3b). No correlation was also observed between the levels of specific IgG4 and those of the specific IgE ($r^2 = 0.035$) (Fig. 3c).

Discussion

In the present study, we prepared a recombinant Der p 2

Table 2. Comparison of r-Der p 2 specific IgE, IgG total and IgG subclass levels between allergic children and controls

		n	Mean (U)	SD	Probability
IgE	Allergic children	240	366.4	383.7	0.0008*
	Controls	25	103.7	137.6	
IgG total	Allergic children	240	737.5	440.3	0.3967
	Controls	25	658.7	450.3	
IgG1	Allergic children	240	445.7	373.3	0.5229
	Controls	25	396.1	324.1	
IgG2	Allergic children	240	472.4	422.3	0.4295
	Controls	25	403.8	298.8	
IgG3	Allergic children	240	635.4	507.8	0.2047
	Controls	25	769.7	448.5	
IgG4	Allergic children	240	879.1	756.7	0.0033*
	Controls	25	420.1	509.2	

* Significant difference.

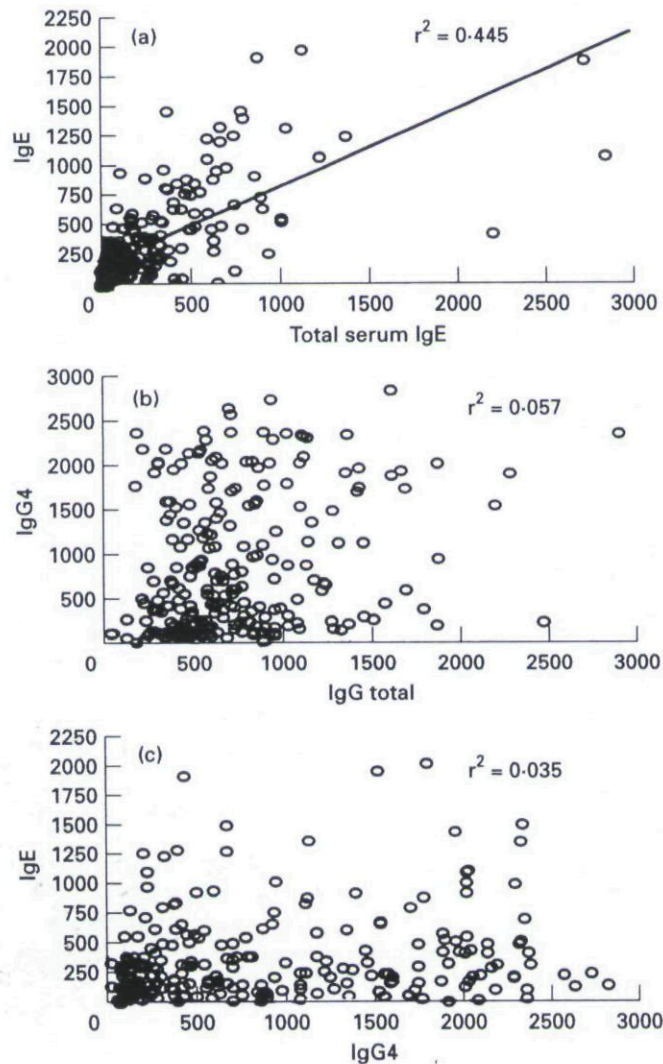


Fig. 3. Scattergrams for correlation studies. (a) Correlation between total serum IgE and specific Der p 2 IgE antibody. (b) Lack of correlation between Der p 2 specific IgG4 and IgG total. (c) Lack of correlation between Der p 2 specific IgE and IgG4.

fusion protein and developed a reliable ELISA system examining levels of antibodies specific for Der p 2 of IgE, IgG total and IgG subclasses.

Specific IgG total and IgG subclass antibodies to several antigens have been measured and analysed in many laboratories. It has been reported that allergen-specific IgG4 antibody increases in patients with hypersensitivity to the corresponding allergen and those with repeated antigenic stimulation such as novice bee keepers or allergen-specific immunotherapy, and that the increases of the specific antibodies of other IgG subclasses are variable [14,18,19,24,25]. In our study, significant differences were found only in the specific IgE and IgG4 antibodies between allergic children and non-allergic controls. There was no statistical difference in levels of the specific antibody of IgG total, IgG1, IgG2

and IgG3 between two groups. This is in agreement with two previous studies: Florine *et al.* reported that the IgE and IgG4 antibodies specific for cat major allergen *Fel d I* [24], and Henrikki *et al.* reported that the mosquito saliva-specific IgE and IgG4 antibodies were detected by immunoblotting [25].

Interleukin-4 is known to be involved in the isotype switching and production of IgE and IgG4 [26]. Thus, one may expect that induction of specific IgE and IgG4 to a certain antigen would be parallel. However, a lack of correlation between the specific IgE and IgG4 as shown in the present study will lead to an idea that mechanisms in induction of the specific IgE and IgG4 are different with each other. In the study of epitopes analysis of Der p 2 antigen, IgE and IgG4 antibodies from patients with mite allergy recognized different epitopes [27]. Some

other regulations may function for induction of IgE and IgG4 antibodies. Alternatively, there are different regulations governing the levels of specific IgE and IgG4 in allergic patients.

A lack of correlation between IgG4 and other IgG subclasses as well as IgGtotal indicates that the induction of specific IgG4 is also independent of other IgG subclasses. This finding is against the study of Nakagawa *et al.* in which the level of IgG4 antibody to house dust mite significantly correlated with those of IgG total. The reasons for this discrepancy are not known. One possibility would be due to the difference in antigen used. The antigen used by Nakagawa *et al.* was crude extract of *Dermatophagoides farinae*, but ours was a purified recombinant Der p 2, a single component of the Der p antigen, thereby the number of epitope being restricted much more than the crude extract. Nevertheless, our present results showing a lack of correlation between IgG4 and IgGtotal or IgG subclasses, will rule out the non-specific binding of IgG4 in our ELISA system.

In conclusion, we demonstrated in the present study that the Der p 2-specific antibodies of IgE and IgG4 but not of other IgG subclasses were increased in allergic children and that the antibody levels of IgE and IgG4 isotypes were not parallel in each individual case. Furthermore, the levels of the specific IgG4 did not correlate with those of other IgG subclasses or IgG total. To understand these phenomena in the mechanisms of allergic inflammation, a serial determination of the specific antibodies of different isotypes in individual allergic children is currently being undertaken.

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