Asian Pacific Journal of Allergy and Immunology (1992) 10 : 11-18

The Change of Allergen-Specific IgG Subclass Antibodies During Immunotherapy in Mite-Sensitive Asthmatic Children

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SUMMARY **Fifty-six *Dermatophagoid farinae* (D.f)-sensitive asthmatic children were hyposensitized by D.f-crude extract for two years. Serum total IgG subclass antibodies and D.f-specific IgE and IgG subclass antibodies were measured by ELISA before and after 2 years of treatment. The resutls showed that 1) After two years of treatment, there were significantly higher levels of total serum IgG1 in both responder and non-responder groups than those before treatment ($p < 0.01$). The responder group also had significantly higher values of total IgG2 and IgG4 after immunotherapy (IT) ($p < 0.05$), but not in the non-responder group. 2) The serum levels of D.f-specific IgG3 and IgG4 antibodies in responder group increased significantly after IT ($p < 0.05$). On the contrary, the D.f-specific IgE and IgG1 IgG1 in the responder group were significantly lower than those before IT. No signi- in the responder group were significantly lower than those before IT. No signi- body titres before and after IT was found in non-responder group. 3) There was a significant correlation between the total IgG4 and D.f-specific IgG4 antibody ($r = 0.634$, $p < 0.01$). The correlation coefficient was 0.634. No correlation was found between the other IgG subclass antibodies and D.f-specific IgG subclass antibodies. 4) Correlations between the levels of D.f-specific IgE and IgG subclass antibodies were highly significant both in IT-responder and non-responder groups. There was a significant correlation between the levels of D.f-specific IgG1 and IgG4 in non-responders, while no relationship was observed in the responder group. In conclusion, an increase of allergen-specific IgG4 and decrease of allergen-specific IgG1 antibodies following immunotherapy can be of clinical benefit to allergic individuals, at least to most children suffering from mite-sensitive asthma.**

The house dust mite, *Dermatophagoid farinae* (D.f) has been identified as one of the main sources of house dust allergens and has been shown to play an important allergenic role in asthmatic children in many countries[1] as well as in Taiwan.[2]

Allergen-specific immunotherapy (IT), formerly also known as desensitization or hyposensitization, was introduced in 1911 by Noon for the treatment of allergic disorders,[3] and has been applied clinically to patients with IgE-mediated allergy to inhaled allergens such as house dust mites, pollens and insect venoms.[4] Clinical improvements associated with IT have been attributed to a variety of immunological mechanisms including the development of IgG "blocking" antibodies, suppressed IgE antibody production, decreased lymphoproliferative response to specific allergen, and decreased mediator release and response.[5-7] Thus, the recognition of four distinct subclasses of IgG has prompted attempts to identify the subclass distribution of such IgG blocking antibodies.

To delineate the working mechanisms of IT, this study was conducted to determine the change of total IgG subclass and specific IgG subclass antibodies against D.f after IT in house dust mites-sensitive asthmatic children.

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MATERIALS AND METHODS

Study populations

This study was approved by the Human Research Committee of National Cheng-Kung University Hospital, and informed consent was obtained from all study subjects. The study population included 56 asthmatic children. The characteristics of the study populations are shown in Table 1. All the patients were sensitive only to mites (*Dermatophagoid farinae,* D.f) as shown by a positive history of exposure, a > 2 + skin test (wheal > 10 mm by intracutaneous route, with mite extract at a concentration of 10^{-6} g/ml, Pharmacia, Uppsala, Sweden) and a positive RAST (Pharmacia Diagnostics AB, Uppsala, Sweden), whereas the control subjects were skin test- and RAST-negative. IT was started with weekly injection of 10^{-6} g/ml of D.f (Pharmacia Co.), the dose was increased gradually until a maximal tolerated dose was reached (usually within 6 months) and then was maintained at 4-week intervals. The total dose of crude mite allergen administered ranged from 126.5 to 219.5 μg. The effectiveness of hyposensitization was evaluated by both the frequency of asthmatic attacks and the amount of medications consumed after IT as compared with those in the year before treatment. In this study, good responders consisted of those patients demonstrating an improvement with > 75% decrease of both parameters.[8] After two years of treatment, 41 out of 56 patients were considered to be good responders. No steroids and bronchodilators were administered for at least two weeks and six hours, respectively, before blood samplings.

Preparation of crude mite extracts

Purified and biologically standardized extract of mites *(D.farinae)* was obtained in a freeze-dried form from Pharmacia AB (Uppsala, Sweden). The mite allergen was extracted according to the method of Haida *et al.*[9] Briefly, 1 g of lyophilized mite powder was defatted with 100 ml ether for 48 hours, then homogenized and stirred continuously in 100 ml phosphate buffered saline for 36 hours. After centrifugation (10,000 × g), the supernatant was lyophilized and stored at -70° C until use. The protein content of the extract was determined by Bio-Rad protein assay kit (BioRad Lab., Richmond, CA), and a final concentration was adjusted to 1 mg/ml (100,000 biological units/ml). The same extract was used for skin testing, immunotherapy, and *in vitro* ELISA.

IgG subclasses

A monoclonal ELISA kit (Zymed Lab. California) was used quantitatively to determine the human IgG subclasses in the serum of asthmatic children. The procedure was performed according to the manufacturer's instruction. Briefly, antibody to human IgG in coating solution (100 μl) was coated onto each well of the microtitre plate and incubated overnight at 37° C. After washing, 100 μl of diluted serum samples (1/2,500, 1/5,000 and 1/10,000) and reference sera were added and incubated at room temperature for 2 hours. Alkaline-phosphatase-conjugated monoclonal antibodies to human IgG1, IgG2, IgG3 or IgG4 were then added to the washed plate and incubated again. Fifty microlitres of the enzyme substrate *p*-nitrophenyl phosphate were then added to react with alkaline phosphatase for 30 minutes. Another 50 μl of 3 M NaOH solution was added to stop the reaction and optical density (OD) was read at 450nm in an ELISA reader (Flow Multiskan, Irvine, U.K.). Each sample was assayed in duplicate.

Assay of the allergen-specific IgE and IgG subclasses antibodies

IgG subclass assay :

Polystyrene microtitre plates were coated with 10 μg/ml of D.f extract in 0.1 M carbonate/bicarbonate buffer, pH 9.6 at 4° C overnight and blocked with 1% fetal calf serum (FCS) in phosphate buffered saline (PBS). Serial dilutions of sera were made duplicates in PBS containing 0.25% FCS and 0.05%

Table 1. Characteristics of study populations

Subjects	No. of cases	Sex M	Sex F	Mean age (years)	Total serum IgE (mean ±SD, μg/ml)
Asthma*					
Responder	41	28	13	11.7	1,662 ± 554
Non–responder	15	10	5	12.3	1,443 ± 634

*Patients who were recently diagnosed and had never received immunotherapy (IT) before.

Tween 20. Mouse monoclonal antibodies (Oxford Ltd, Basingstoke, England) to human IgG1 (HP6012, NL16), IgG2 (HP6008, COM1), IgG3 (HP6010, ZG4) and IgG4 (HP6013, GB7b) at 1/5,000 dilution were added to appropriate rows, followed by an affinity-purified, human IgG-absorbed, peroxidase-conjugated antiserum to mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) at 1/10,000 dilution. The plates were washed between each step with tap water, and all incubations were carried out for 1 hour at 37°C. Finally, 0.4 mg/ml of *o*-phenylenediamine (Sigma) and 0.1 µl/ml of H_2O_2 were added to each well for 20 minutes. The reaction was stopped by adding 50 µl of 2N H_2SO_4 to each well and OD was read at 492 nm in a Titertek Multiskan (Flow Laboratories, Rickmansworth, England). Coated wells to which only PBS-BSA had been added served as the background control and their values were subtracted from those of the test samples. All assays were performed at least in duplicates, and the experimental error was up to 5% when calculated from triplicate determinations of the same serum samples. The mean absorbance of duplicate estimations was expressed as percent of that of the control serum reading from a healthy individual who is not sensitive to D.f (negative reference) using the following formula :

$$\text{Specific IgG level (\%)} = \frac{\text{sample reading - background reading}}{\text{negative reference reading - background reading}} \times 100$$

IgE assay :

The D.f-specific IgE antibody titre was determined by ELISA method as described above except with a mouse monoclonal antibody to human IgE at 1/3,000 dilution.

Statistical analysis

The data obtained were analyzed statistically by paired Student's *t* test. The significant threshold was fixed at 0.05.

RESULTS

The 56 patients studied were divided into two groups: responders and non-respodners according to their symptoms of asthma and medications consumed after IT. The responder group consisted of 41 patients and the non-responder group of the remaining 15 patients.

The serum IgG subclass concentrations in asthmatic children who had undergone a course of immunotherapy are listed in Table 2. After two years of treatment, there were significantly higher values of total serum IgG1 in both responder and non-responder groups than those before IT ($p < 0.01$, Fig. 1). The responder group also had significantly higher values of total serum IgG2 and IgG4 after immunotherapy ($p < 0.05$), whereas the non-responder group showed no significant changes of total serum IgG2 and IgG4 levels after IT. All the samples were run simultaneously. The coefficient of variation of intraassay for IgG subclass levels in patients' sera was lower than 9.0%.

Table 3 shows the mean values of D.f-specific IgE and IgG subclass antibody titres in the asthmatic children. The results showed that the serum levels of D.f-specific IgG3 and IgG4 antibodies in responder group increased significantly after IT ($p < 0.05$). On the contrary, the D.f-specific IgE and IgG1 in responder group were significantly lower than those before IT ($p < 0.05$, Fig. 2). No significant difference in

Table 2. Serum IgG subclass concentrations in the asthmatic children during immunotherapy (IT)

IgG (mg/ml)	Responder (n=41)		Non–responder (n=15)	
	Pre–IT	Post–IT	Pre–IT	Post–IT
IgG1	3.58 ± 1.09#	4.16 ± 1.42*	3.45 ± 1.09	3.91 ± 1.67*
IgG2	1.33 ± 0.45	1.76 ± 0.76*	1.22 ± 0.67	1.45 ± 0.87
IgG3	0.43 ± 0.28	0.49 ± 0.54	0.30 ± 0.16	0.35 ± 0.23
IgG4	0.25 ± 0.11	0.38 ± 0.11*	0.23 ± 0.14	0.25 ± 0.14

#Mean ± SD values (mg/ml) of serum IgG subclass.
*Significant difference ($p < 0.05$) was noted as compared with those before IT.

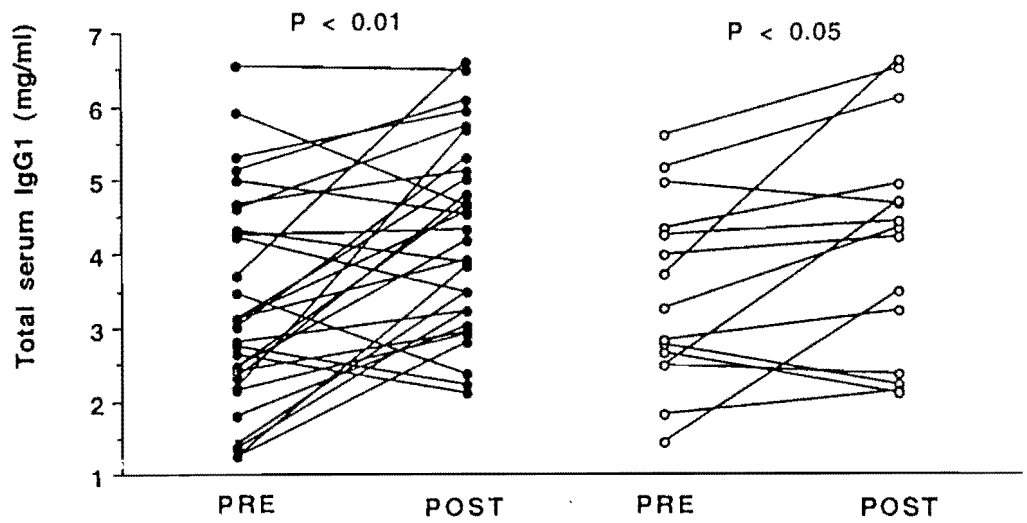

Fig. 1 Changes of serum total IgG1 antibody levels in responder (solid circle) and non–responder (open circle) asthmatic children before (PRE) and after (POST) immunotherapy.

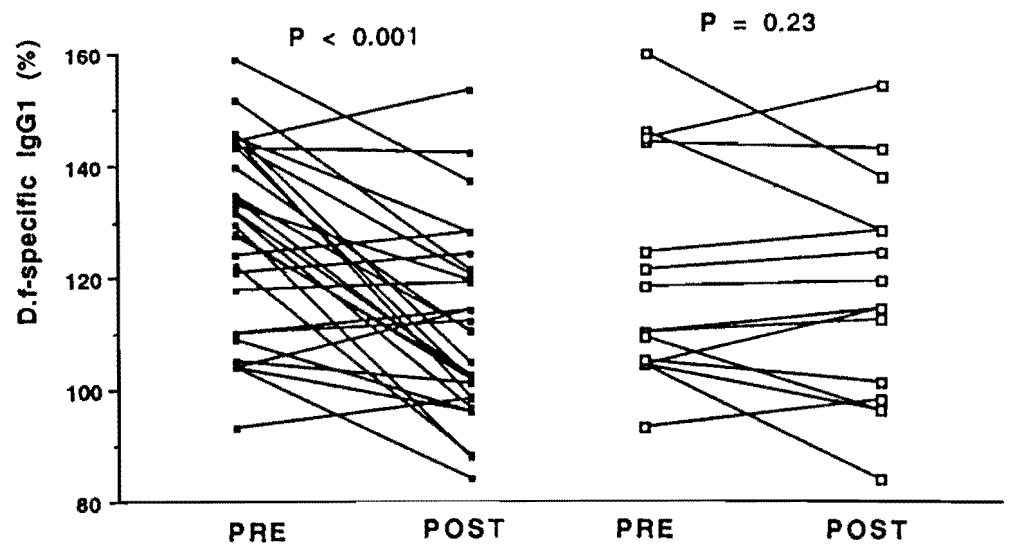

Fig. 2 Changes of specific IgG1 antibody levels to *D. farinae* in responder (solid square) and non–responder (open square) mite–sensitive asthmatic children before (PRE) and after (POST) immunotherapy.

Table 3. D. f–specific IgE and IgG subclass antibody titres in the asthmatic children during immunotherapy.

	Responder (n=41)		Non–responder (n=15)	
%	Pre–IT	Post–IT	Pre–IT	Post–IT
IgE	128.1 ± 7.9#	102.9 ± 15.0*	116.1 ± 18.9	114.8 ± 19.4
IgG1	131.4 ± 16.4	102.0 ± 13.5*	119.6 ± 19.5	116.5 ± 19.3
IgG2	160.9 ± 67.4	152.6 ± 76.4	167.6 ± 37.6	163.4 ± 21.7
IgG3	132.1 ± 35.9	168.7 ± 25.6*	137.1 ± 44.1	146.8 ± 51.9
IgG4	137.9 ± 50.6	191.8 ± 39.8*	147.8 ± 40.6	152.1 ± 34.4

#Mean ± SD values of D. f–specific antibody titres

*Significant difference ($p < 0.05$) was noted as compared with those before IT.

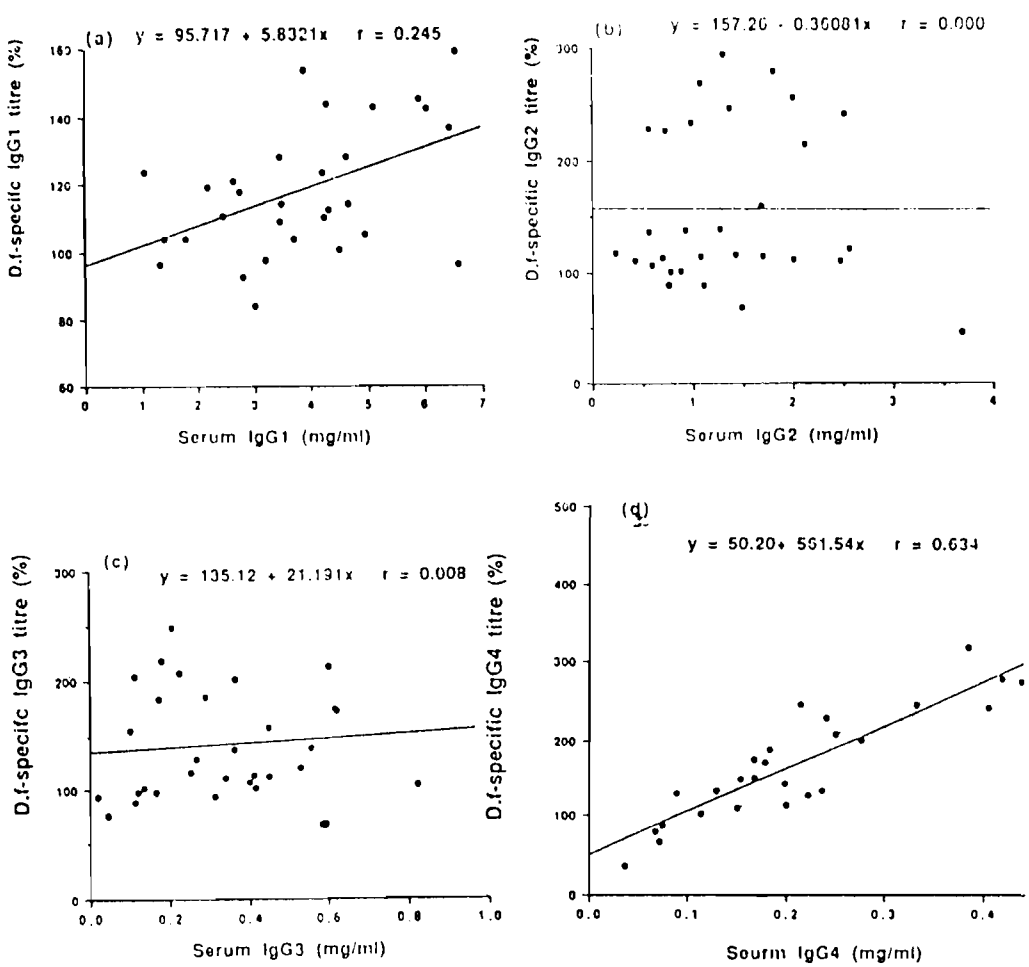

Fig. 3 Correlation between total serum IgG subclass (igG1, IgG2, IgG3 and IgG4) antibody levels and IgG subclass antibody titres specific to *D. farinae* in mite sensitive asthmatic children after IT.

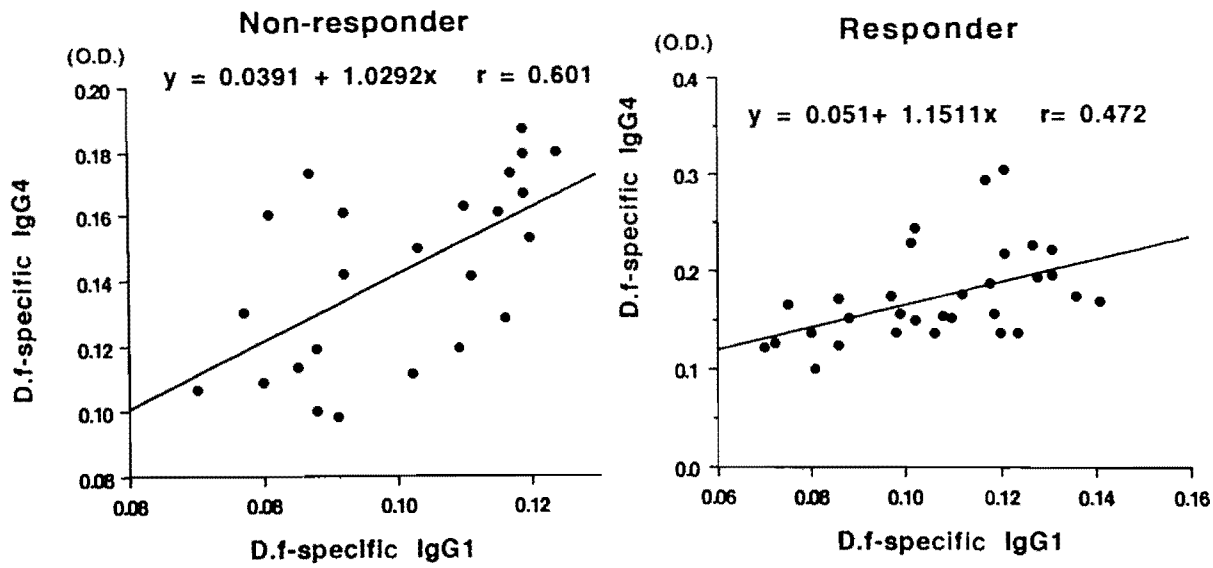

Fig. 4 Correlation between the levels of D. f–specific IgG1 and IgG4 antibody titres in responder and non–responder groups.

Table 4. Correaltion between the level of D. f–specific IgE and IgG subclass antibodies in asthmatic children

Correlation (r)	Responder	Non–responder
IgE vs. IgG1	0.768	0.673
IgE vs. IgG2	0.764	0.751
IgE vs. IgG3	0.993	0.881
IgE vs. IgG4	0.859	0.785

the mean values of the D.f-specific IgE, IgG and IgG subclass antibody titres before and after IT was found in non-responder group.

The correlation between total and D.f-specific IgG subclass antibody levels in 30 non-selected asthmatics is shown in Fig. 3. There is a significant correlation between the total IgG4 and D.f-specific IgG4 antibody levels ($p < 0.01$). The correlation coefficient was 0.634. No correlation was found between the other three IgG subclass antibodies and the same D.f-specific IgG subclass antibodies.

Correlations between the levels of D.f-specific IgE and IgG subclass antibodies were highly significant both in IT-responder and non-responder groups (Table 4). There was significant correlation between the levels of D.f-specific IgG1 and IgG4 in non-responders ($r = 0.601$, $p < 0.01$), while no relationship was observed between these two subclass antibodies in the responder group ($r = 0.472$, $p > 0.05$) (Fig. 4).

DISCUSSION

Although immunotherapy (IT, or hyposensitization) has been used widely for nearly 80 years and has been shown to be effective in treating respiratory allergies, its working mechanism(s) still remains unknown.[5] Its clinical effect seems to be due to the production of IgG-blocking antibodies or the reduction of IgE antibodies. Many attempts have been made recently to identify the IgG subclass antibodies, which are induced by immunotherapy as efficient blocking antibodies.[10-12]

D.farinae (D.f) is one of the main components of house dust and is a major allergen producing bronchial asthma and allergic rhinitis all over the world[1] including Taiwan.[2] In this study, we used two assay procedures to measure the serological

changes of IgG subclass antibody and D.f specific-IgG subclass and IgE antibody titres during the course of IT. The results revealed that the serum IgG1 antibodies increased in both responder and non-responder group after IT, but the serum IgG2 and IgG4 subclass antibodies increased after IT only in the responder group. In addition, the D.f-specific IgG3 and IgG4 increased, while D.f-specific IgG and IgG1 antibodies decreased after IT in the responder group. The D.f-specific IgE and IgG subclass antibodies did not change statistically in non-responder group.

There is evidence to suggest that IgG4 antibodies are synthesized in response to chronic exposure to antigen and repetitive specific allergen injections.[13,14] However, the role of antibodies of the IgG4 subclass in human allergic disease is controversial. Studies of the subclass of the IgG antibody responses during the period of 2 years of IT with timothy grass pollen suggested that only IgG1 and IgG4 responses were induced.[15] The results of this study were in consistent with those previously studied by us[16] and by Tsai's group[17] in that the IT treated patients showing a dominant increase in IgG4 antibody, particular those with a simultaneous decrease of IgE antibody, had a better response.

We also found that there was a significant correlation between serum IgG4 and D.f-specific IgG4 antibody ($r = 0.634$), while there was no significant correlation between the other three serum IgG subclass antibodies and the same D.f-specific IgG subclass antibodies. Because the serum total IgG1 antibody increased, while D.f-specific IgG1 antibody decreased after IT in responder group, these results may suggest that D.f-specific IgG1 has been involved in the allergic mechanism. Moreover, there was significant correlation between the levels of D.f-specific IgG1 and IgG4 in non-responders ($r = 0.601$, $p < 0.01$), while no relationship was observed between these two subclass antibodies in the responder group ($r = 0.472$, $p > 0.05$). It has been reported that there is a close correlation between the presence of high titres of IgG1 antibody and the propensity of late asthmatic response (LAR) to develop in house dust mite-sensitive asthma.[18] The role of IgG1 antibody in allergic reaction remains much to be elucidated. It seems likely that all subjects, including non-atopic people, can recognize and produce IgG1 antibodies to inhaled and food allergens.[19] This IgG1 antibody response seems to decline after reaching a certain level of allergen exposure. However, it is possible that IgG1 antibodies may become gradually harmful to the organism as their level increases.[20]

In conclusion, an increase of allergen-specific IgG4 and a decrease in specific IgG1 subclass antibodies following immunotherapy can be of clinical benefit to allergic individuals, at least to most children suffering from mite-sensitive asthma. The change of total serum IgG subclass and allergen-specific IgG subclass antibodies induced by immunotherapy may provide objective assistance to the control of specific treatment.

ACKNOWLEDGEMENTS

We thank Zan-Shan Tao and Chih-San Liu for their assistance. This work was supported partly by NCKUH Research Funds 80-021.

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