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Specific IgG1 and IgG4 Antibodies to Citrus Red Mite in Citrus Farmers: A Study of Their Relationship to Respiratory Symptoms

Citrus red mite (CRM) is known as the most common sensitizing allergen in subjects with asthma and rhinitis working on citrus farms. The aim of this study is to evaluate the role of specific IgG1 (sIgG1) and specific IgG4 (sIgG4) to CRM in citrus farmers. Questionnaire survey and skin prick test including CRM antigen was done by 136 workers. Specific IgE (sIgE), sIgG1 and sIgG4 to CRM were detected by enzyme-linked immunosorbent assay (ELISA). CRM-sensitive-asthma was diagnosed upon presence of asthmatic symptoms by questionnaire, airway hyperresponsiveness to methacholine and sIgE to CRM. CRM-sensitive rhinitis was diagnosed upon presence of rhinitis symptoms and sIgE to CRM. Eleven (8.1%) had CRM-sensitive asthma and 25 (18.4%) had CRM-sensitive rhinitis. Significant association was noted between presence of asthmatic symptoms and sIgE or sIgG4 ($p<0.05$, respectively), while no significant association was noted in sIgG1 ($p>0.05$). Significant association was noted in the prevalence between sIgG4 and sIgE ($p<0.05$), while no significant association was noted between sIgG1 and sIgG4 or sIgE ($p<0.05$, respectively). There was a significant correlation between sIgE and sIgG4 level ($r=0.39$, $p<0.05$). These findings suggest that the presence of sIgG1 to CRM is response to CRM exposure, and further studies will be needed to evaluate the role of sIgG4.

Key Words: *Citrus Red Mite; Specific IgG1; Specific IgG4; Specific IgE; Asthma; Rhinitis; Sencitivity and Specificity; IgG; IgE*

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INTRODUCTION

Spider mites (Family Tetranychidae) can be pests to citrus products throughout the world, especially in subtropical areas (1); the two most common species are the citrus red mite (*Panonychus citri*, CRM) and the Texas citrus mite (*Eutetranychus annecki*). CRM, a mite which causes a lot of damage to citrus, rapidly increases in population that can be harmful in early spring, with two peak periods in late spring and mid-fall (1). Cheju Island is located off the southern part of the Korea mainland, and has a population of about half a million people. Citrus trees were introduced before 1945, and citrus fruit has been one of the main crops for about 35 years. About one hundred thousand adults are involved in commercial citrus cultivation.

In an occupational environment, the non-pyroglyphid storage mites are often found to cause allergic problems (2, 3), though pyroglyphid mites, or *Dermatophagoides*, are chiefly responsible for house dust allergy (4). There have been several reports of occupational allergy to Tetranychidae species (5-7). We reported 16 cases of CRM-induced asthma among farmers on citrus orchards (8). Allergy skin prick test study of citrus farmers showed that CRM was the most common sensitizing allergen with a higher sensitization rate than house dust mite (9). The role of sIgG in an occupational setting seems to be complicated. There has been no data concerning the role of serum sIgG antibody to CRM in CRM exposed farmers.

In this study, we evaluated the role of sIgG1 and sIgG4 by enzyme-linked immunosorbent assay (ELISA) in 136 CRM-exposed farmers and their relationship with rhinitis and asthmatic symptoms. The relationship with sIgE was also investigated.

MATERIALS AND METHODS

Study subjects

One hundred and thirty-six subjects involved in citrus production on Cheju Island were enrolled in this study. They had worked on citrus orchards for a mean period of 23.7 (range 5-40) yr; 72 were males and 64 were fe-

males, with mean age of 47.6 (range 18-87) yr; 51 subjects (37.5%) had a history of smoking. They responded to a questionnaire, administered by physicians and allergists, and underwent skin prick tests (SPT) involving CRM extracts and 11 common inhalant allergens (*D. pteronyssinus*, *D. farine*, mugwort, tree pollen mixture I and II, ragweed; grass pollen mixture, *Aspergillus* spp., and *Alternaria* spp., animal epithelium, cockroach, Allergopharma Co., Hamburg, Germany). Subjects who had asthmatic symptoms during the previous 12 months were evaluated by methacholine bronchial provocation test, which was performed by the method described previously (8, 9). Diagnosis of asthma was based on presence of asthmatic symptoms, serum specific IgE to CRM and positive response on methacholine bronchial challenge test and classified as Group I. The farmers having rhinitis symptoms alone without asthmatic symptoms, but having high specific IgE antibody to CRM was classified as Group II. The other exposed farmers were classified as Group III. Sera from the 136 subjects were collected and stored at -20℃. The subjects gave their informed consent as regulated by Seoul National University Hospital, Seoul, Korea.

Questionnaire survey

Physicians together with allergists administered a questionnaire, which was developed from the Respiratory Disease Questionnaires of the American Thoracic Society (10). It was translated into Korean and included questions regarding demographic data and smoking, occupational and medical history. The medical history concerned asthmatic symptoms (paroxysmal cough, wheezing and nocturnal dyspnea or dyspnea at rest) and nasal symptoms (sneezing, watery rhinorrhea and nasal obstruction), and the temporal relationship between exposures at workplace and aggravation of symptoms. According to the questionnaire, if subjects had experienced two or more asthmatic and/or nasal symptoms, they were assumed to be positive.

Preparation of CRM extracts

CRMs were provided by the Citrus Research Center on Cheju Island. They were extracted with phosphate-buffered saline [(PBS, pH 7.5), 1:5 w/v] at 4℃ for 1 hr followed by centrifugation at 5,000 rpm. The supernatants were passed through a syringe filter (MSI, U.S.A.), and for SPT, the 1:5 w/v extract was mixed with an equal amount of sterile glycerin. The supernatant was dialyzed (the cut-off molecular weight was 6,000 Da) against 4 L of distilled water at 4℃ for 48 hr, and lyophilized at -70℃ for antigen preparation used in ELISA.

ELISA for specific IgE antibodies to CRM extracts

The presence of specific IgE antibody to CRM was determined by ELISA. Microtitre plates (Dynatech, UA, U.S.A.) were first coated with 100 μL/well of CRM (1 μg/well) extracts, and left at 4℃ overnight. Each well was washed 3 times with 0.05% Tween phosphate buffered saline (PBS-T), and the remaining binding sites were blocked by incubation with 350 μL of 3% bovine serum albumin (BSA)-PBS-T for 1 hr at room temperature. The wells were then incubated for 2 hr at room temperature with 50 μL of either the patient or control sera (undiluted) from 30 asthmatic patients showing a negative SPT response to common inhaled allergens as well as to CRM. After washing three times with PBS-T, 50 μL of the 1:500 v/v biotin-labeled goat anti-human IgE antibody (Vector Co., Berlingham, CA, U.S.A.) was added to the wells and incubated for 1 hr 30 min at room temperature. The wells were then washed three times with PBS-T and incubated with 1:1,000 v/v streptovidin-peroxidase (Sigma Co., St. Louis, U.S.A.) for 30 min before another washing cycle which was followed by incubation with 100 μL of ABTS (2.2'-azinobis-3-ethyl-benzthiazoline sulfuric acid in a citrate phosphate buffer) for 10 min at room temperature. The reaction was stopped by adding 100 μL of 2.5 N sulfuric acid and the absorbance was read at 490 nm by an automated microplate reader. All assays were performed in duplicate. The cut-off value (0.24) of the positive IgE binding was determined from the mean plus two folds standard deviation of the absorbance value from 30 control subjects.

ELISA for specific IgG1

Fifty microlitres of diluted patient serum or negative control serum (1/200 in diluent buffer; PBST containing 3% BSA) was added to each well coated with CRM. After incubation for 2 hr at 25℃, the wells were washed three times with PBST. One hundred microlitres of horseradish peroxidase (HRP)-conjugated goat anti-human IgG1 (Sigma Co., U.S.A.) diluted into 1/2,000 v/v with 3% BSA-PBS-T was added to each well. The plates were then incubated at 4℃ for 2 hr. The wells were washed three times with PBS-T and then 50 μL of substrate solution was added, containing 0.01 M *o*-phenylenediamine-HCl in citrate phosphate buffer, pH 4.2, supplemented with 0.012% H_2O_2 before use. After incubation for 15 min at room temperature, 50 μL of 1 N H_2SO_4 was added to stop the reaction. The optical density of the solution at 490 nm was determined with a microtitre plate reader (MR 600, Dynamic Product, U.S.A.). The antibody levels were expressed as absorbance at 490 nm. The positive cut-off value was deter-

mined from the mean absorbance and two-fold standard deviation of 30 negative controls (mean $+2\times SD = 0.11$). All final absorbance values were obtained by subtracting absorbance from each uncoated well.

ELISA for specific IgG4

Fifty microlitres of patient serum or negative control serum (undiluted) was added to each well coated with 1 μg/well of CRM, and incubated for 3 hr at room temperature. After the wells were washed three times with PBS-T, 50 μL of biotin-conjugated mouse monoclonal anti-human IgG4 (Sigma Chemical Co., U.S.A.) diluted to 1/1,000 (w/v) with 3% BSA-PBST was added; they were incubated for 1 hr 30 min at room temperature. The wells were washed three times with PBS-T. Then, 50 μL of 1/1000 diluted streptavidin-HRP (Sigma Chemical Co., U.S.A.) was added and incubated for 30 min. The wells were washed five times and 100 μL of substrate solution, tetramethybenzidine (TMB) was added to each well. After incubation for 10 min, 100 μL of 2.5 N sulfuric acid was added to stop the reaction. The absorbance was measured at 450 nm with a microplate reader, and the antibody levels were expressed as absorbance. The cut-off value was determined as 0.14 from the mean absorbance +2 S.D. of 30 controls.

Statistical analysis

ANOVA were applied using SPSS version 7.0 (Chicago, U.S.A.) to evaluate the statistical differences among the three groups. Pearson correlation analysis was applied to evaluate the relationship between two values. p value of 0.05 or less was regarded as significant.

RESULTS

Prevalence of CRM-sensitive asthma and rhinitis

Of the 45 subjects who complained of asthmatic symptoms, 11 (8.1%) had CRM-sensitive asthma. The mean duration of the asthmatic symptoms was 6.4 (range 1-20) yr. Sixty-four subjects experienced recurrent nasal symptoms. Twenty-five had CRM-sensitive rhinitis alone and nine of Group I subjects had rhinitis symptom. The prevalence of atopy defined by positive skin prick test responses (A/H ratio $\geq$ 1.0) to more than one common inhalant allergen was 17.1%, and when citrus red mite was included, 25.9% of the farmers showed positive responses. All the subjects classified to Group I and II showed positive responses to citrus red mite on skin prick test.

Specific IgG1 and specific IgG4 antibodies to CRM, and its relationship to nasal and asthmatic symptoms

When an ELISA was considered positive (>0.105 of absorbance value for sIgG1, >0.13 of absorbance value for sIgG4), 23 (16.9%) subjects were found to have sIgG1 and 22 (16.2%) with sIgG4, with sIgE was detectable in 54 (39.7%) farmers. No significant differences were noted in prevalences of sIgG1 and sIgG4 according to smoking or atopic status ($p>0.05$, respectively, data is not shown). Table 1 summarizes the prevalences of sIgE, sIgG and sIgG4 antibodies to CRM according to presence of rhinitis and asthmatic symptoms in exposed farmers. Of the symptomatic farmers complaining of nasal and asthmatic symptoms, 34 (53.1%) and 26 (57.8%) had high sIgE bindings respectively,

Table 1. Association between rhinitis and asthmatic symptoms, and prevalence of specific IgE, IgG1 and IgG4 antibodies

Specific antibodies	Rhinitis symptoms		Asthmatic symptoms	
	Presence	Absence	Presence	Absence
Specific IgE*				
Positive	34	20	26	28
Negative	30	52	19	63
Specific IgG1†				
Positive	13	10	11	12
Negative	51	62	34	79
Specific IgG4†				
Positive	14	8	14	8
Negative	50	64	31	83

*$p<0.05$ for rhinitis symptoms, $p<0.05$ for asthmatic symptoms

†No significant differences were noted in prevalences of sIgG1 whether rhinitis and asthmatic symptoms were present or not ($p>0.05$, respectively)

‡$p>0.05$ for rhinitis symptoms, $p<0.05$ for asthmatic symptoms

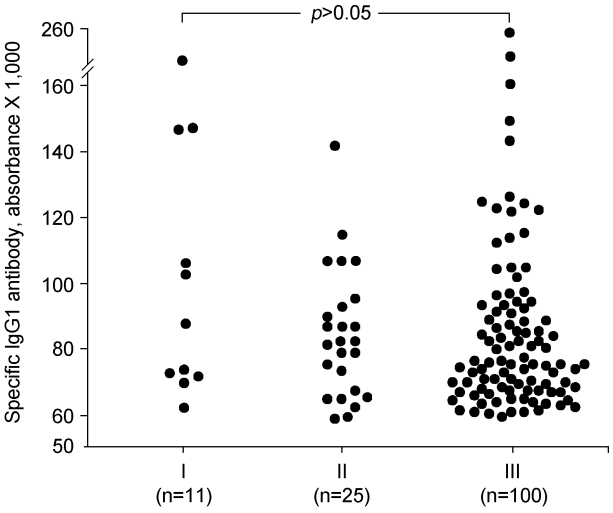

Fig. 1. Specific IgG1 bindings to citrus red mite in the three groups. No significant differences are noted among the three groups ($p>0.05$). Group I, CRM-sensitive asthma; Group II, CRM-sensitive rhinitis; Group III, Controls

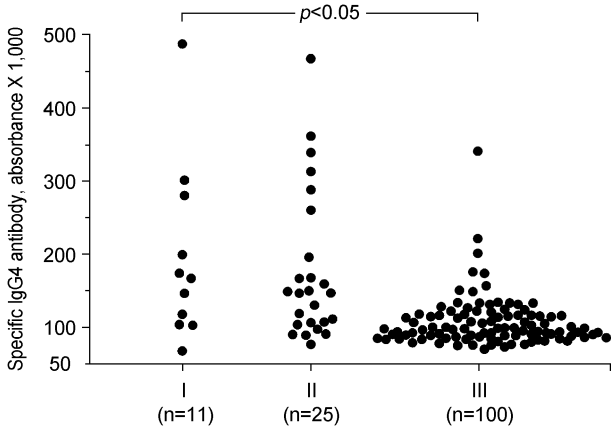

Fig. 2. Specific IgG4 bindings to citrus red mite in the three groups. Significant difference is noted among the three groups ($p<0.05$). Group I, CRM-sensitive asthma; Group II, CRM-sensitive rhinitis; Group III, Controls

whereas 20 (27.8%) and 28 (30.8%) out of asymptomatic farmers were positive according to statistical significance ($p=0.003$). Although there was no significant association between presence of nasal symptoms and sIgG4 ($p>0.05$), a significant association was noted between presence of asthmatic symptoms, and prevalence of IgG4 antibodies ($p<0.05$). No association was found between the presence of rhinitis and asthmatic symptoms, and the prevalence of sIgG1 antibody with 11 (24.4%) of 45 subjects demonstrating asthmatic symptoms and 12 (13.2%) of 91 asymptomatic farmers revealing sIgG1 antibody to CRM ($p>0.05$, respectively).

Specific IgE, IgG and IgG4 to CRM according to diagnosis

Fig. 1 shows sIgG1 bindings to CRM in three different groups. No significant difference was noted in the prevalences of sIgG1 among the three groups ($p>0.05$); four (36.4%) in Group I, 5 (20%) in Group II and 14 (14%) in Group III had high sIgG1. Fig. 2 shows sIgG4 bindings in three different groups. Six (54.5%) in Group I had high sIgG4 antibody, followed by Group II (40%) and Group III (6%) with statistically significant difference among the three groups ($p<0.05$).

Association between specific IgE and IgG4 antibodies

Table 2 demonstrates association between prevalences of sIgE and sIgG1 or sIgG4 antibodies. Significant association was noted between sIgE and sIgG4 ($p<0.05$). No association was noted between sIgE and sIgG1 ($p>0.05$). Significant correlation was noted between sIgE and sIgG4 levels ($r=0.39$, $p<0.05$, Fig. 3A), while poor correlation was noted between sIgE and sIgG1 ($r=0.14$, $p>0.05$, Fig. 3B).

DISCUSSION

We recently reported 16 cases of CRM-induced asthma, which may be an IgE-mediated response, confirmed by bronchial challenge with CRM extract in citrus farmers (8). It has been suggested that CRM is one of the major causative allergens in the development of adult-onset asthma in Cheju Island, as the prevalence of asthma among citrus-cultivating adults was 12.1%, higher than the 4.6% found on the Korean mainland (11). The pre-

Table 2. Association between specific IgE, IgG1 and IgG4 antibodies

Specific antibodies	Specific IgG1 antibody*		Specific IgG4 antibody†	
	Positive	Negative	Positive	Negative
Specific IgE				
Positive	13	41	20	34
Negative	10	72	2	8

*$p>0.05$, †$p<0.001$

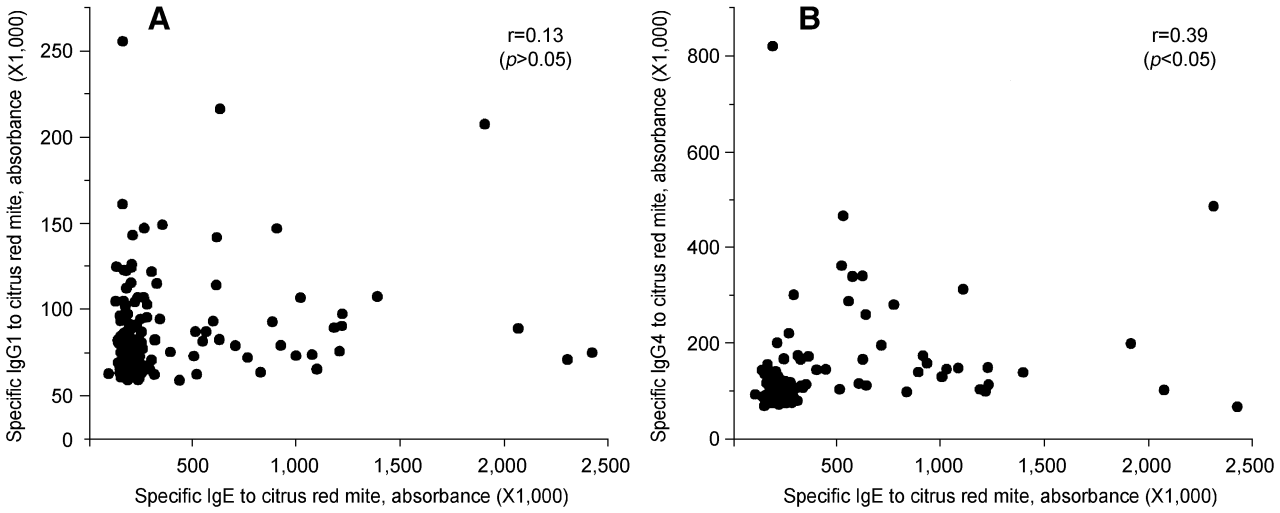

Fig. 3. Correlation between specific IgE and IgG4 (A) and IgG1 (B) antibodies to citrus red mite.

valence of asthma was also higher in subjects with positive skin response or specific IgE antibody to CRM than in those without skin response or specific IgE antibody (9).

The pathogenetic mechanism of CRM-induced asthma is still under investigation. It was suggested that IgE-mediated reaction may induce rhinitis and asthma in workers exposed to CRM (8, 9). The subjects with upper and/or lower respiratory symptoms among citrus farmers had a higher sensitization rate and serum specific IgE antibodies to CRM than to those without it (9). The asthmatic workers showed strong positive responses to CRM on allergy skin prick test and also showed high serum specific IgE antibody. Bronchoprovocation test with CRM extract showed immediate and/or late asthmatic responses. However, one asthmatic subject, without sIgE, was found to have high sIgG4. We speculate that another humoral immune mechanism such as specific IgG-mediate reaction may possibly be involved in CRM-induced asthma.

There has been no report concerning clinical significance of sIgG1 or sIgG4 antibodies in CRM-induced asthma and/or rhinitis. The sIgG response in other occupational asthma studies seems to be complicated, and has elicited variable results according to different antigens. Previous studies on isocyanate asthma (12, 13) have demonstrated that the level of sIgG to isocyanate (hexamethylene diisocyanate, methylene diisocyanate and toluene diisocyanate) showed an association with the results of specific inhalation challenges, while the levels of specific IgE did not. Forster et al. (14) reported that sIgG to tetrachlorophthalic anhydride (TCPA) was detected in all asthmatic workers and some of the exposed asymptomatic workers. Moreover, the sIgG antibody increased in five out of seven occupational asthmatic workers due to TCPA, but was not detected in any of the asymptomatic individuals. Our previous study (15) on reactive dye asthma revealed that the prevalence of sIgG and sIgG4 was significantly higher in symptomatic workers complaining of asthmatic symptoms. These findings support the view that sIgG may contribute to the development of respiratory symptoms in exposed workers.

However, several studies in other occupational settings have suggested that the presence of sIgG may represent a response to high-dose exposure and not directly related to the development of respiratory symptoms. A study by Quirce et al. (16) on carmine dye exposed workers showed that all employees of the factory, regardless of their occupation or whether they had symptoms, had high levels of sIgG, probably as a consequence of the highly carmine-contaminated environment to which they were exposed. Similarly, a study of sIgG in workers at potato-processing industry (17) and grain dust industry (18) showed that sIgG was found in nearly all the workers, and specific IgG4 was found in about half of the workers. In that study, the possibility of sIgG4 as a blocking antibody was reported, in which the level of sIgG4 in symptomatic workers tended to be lower in non-symptomatic workers. In this study, we measured serum sIgG1 and sIgG4 antibodies to CRM by ELISA in exposed farmers. The prevalence of sIgE in symptomatic workers was the highest followed by sIgG1 and sIgG4 antibodies in exposed farmers. Regarding the relationships with respiratory symptoms, higher sIgG4 antibody as well as sIgE were detectable in subjects with asthmatic symptoms than in asymptomatic subjects. No significant differences were noted in the prevalences of sIgG1. Significant association was noted between the prevalence of sIgE and sIgG4. Most of the IgE antibody responders also had sIgG4 antibody with significant

correlation between sIgE and sIgG4 levels. The level of specific IgG4 antibody in subjects with high specific IgE was significantly higher those without specific IgE. A significant correlation was noted between specific IgE and IgG4 antibody. These findings suggest that sIgE and sIgG4 antibodies to CRM might be a parallel immune response to CRM as other investigators have reported in grass pollen and mite allergen (19-21). Another separate finding of this study was the lower prevalence of sIgG compared to those of other occupational studies, especially higher molecular weight agents (17, 18). This may be due to relatively low exposure to CRM since the subjects were intermittently exposed; farmers worked outdoors and exposure to CRM was intermittent and seasonal, since exposure to CRM had two peak periods in late spring and mid-fall and were minimal during other seasons, especially winter, which may have resulted in a latent period for CRM-sensitive asthmatic patients of longer than 10 years.

In conclusion, the presence of sIgG1 to CRM is derived from response to CRM exposure and further studies will be needed to confirm the role of sIgG4 in the development of upper and lower respiratory symptoms induced by CRM in exposed farmers.

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