The effect of cosensitization with buckwheat flour on the production of house dust mite-specific IgE

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Written by Youn Ho Shin

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ABSTRACT

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Rationale: There are several studies reporting that food sensitization in early infancy increases the risk of sensitization to inhalant allergens later in life. To evaluate whether cosensitization with buckwheat (BW) flour has any effect on the production of house dust mite (HDM)-specific IgE, we performed a study using our murine model of BW allergy.

Methods: C3H/HeJ mice (4 weeks, female) were sensitized intraperitoneally with HDM mixed with $Al(OH)_3$ on day 1, followed by 4 times of intranasal sensitizations (on days 14, 15, 16, and 21). Group 1 mice were cosensitized intragastrically with

BW/cholera toxin (CT) (on days 0, 1, 2, 7, and 18) during sensitization with HDM, group 2 mice were cosensitized intragastrically with CT only (on days 0, 1, 2, 7, and 18) and group 3 mice were used as naïve controls. HDM- and BW-specific IgE levels and antigen specific T-cell proliferation and cytokine productions were evaluated.

Results: In group 1, BW-specific IgE levels were highest at week 4, and the HDM-specific IgE levels were highest at week 3, respectively (98.45 ± 64.37 ng/mL, 169.86 ± 55.54 ng/mL). In group 2, HDM-specific IgE levels reached a peak at week 3 and was remarkably higher (810.52 ± 233.29 ng/mL) compared to that of group 1 mice (169.86 ± 55.54 ng/mL). The IL-4 and IFN-γ levels in the HDM-stimulated culture supernatants of splenocytes were not significantly different among groups 1, 2, and 3.

Conclusion: In the current study using a murine model of BW allergy, we postulate that the cosensitization with BW may down-regulate the specific IgE response to HDM sensitization.

Key words: murine model, buckwheat, house dust mite, IgE, cosensitization

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I. INTRODUCTION

Food allergy is now accepted as a major health problem worldwide, especially in Westernized nations. Recent studies suggest that food allergies affect 8% of children and 2% of adults, implying that it primarily is a disease of children. Food allergies are adverse immunologic reactions that might be due to IgE- or non-IgE-mediated immune mechanisms and are known to cause gastrointestinal allergy, atopic dermatitis, urticaria, and respiratory allergic diseases. Although intact food antigens routinely penetrate into the gastrointestinal tract, clinical symptoms of allergy such as

vomiting, diarrhea, and urticaria rarely develop, because tolerance to food antigens is established in most individuals. However, there are individuals with atopic tendency who become sensitized to several food antigens such as milk, egg, wheat, soybean, peanut, buckwheat, etc, and ultimately develop allergic symptoms.[1, 2]

Buckwheat (BW) (*Fagopyrum esculentum*) is a major cause of food hypersensitivity in children in Korea and Europe. In 1909, Smith first described a young patient who presented with severe symptoms of asthma, allergic rhinitis, urticaria, and angioedema after ingestion of a small quantity of buckwheat.[3] House dust mite is the most important aeroallergen worldwide,[41] and 70% of pediatric pulmonologic patients and half of adult patients in Korea are known to have been sensitized to house dust mite, *Der f* and *Der p*, based on skin tests.[4] Recent clinical studies have revealed that children with sensitization to food allergens in early infancy are at a higher risk of developing sensitization to inhalant allergens later in life compared to those without sensitization to food allergens.[5-11,43,44] Nickel *et al.* showed that risk factors for sensitization to indoor and/or outdoor allergens at the age of 3 years were 1) a positive family history and 2) the presence of hen's egg-specific immunoglobulin (Ig) E antibodies (≥ 0.35 kU/L) at the age of 12 months.[10] In the same cohort, twice as many children who, at the age of 7 years, presented with bronchial hyperresponsiveness (BHR) and asthma were sensitive to food allergens at the age of 1 year compared with nonasthmatic children.[12] Furthermore, those children who were persistently sensitized for more than 1 year to food allergens had a 5.5-fold

higher risk of developing asthma than children who were only temporarily food sensitized.[5] Rhodes *et al.* also have conducted a birth cohort study in which they asserted that sensitization to dietary allergens occurred in infancy and waned after early childhood, but it predicted the early sensitization to inhalant allergens and persistence of asthma up to the age of 22 years.[11] Taken together, sensitization to food allergens early in life is thought to be associated with development of IgE-mediated hypersensitivity to inhalant allergens and of allergic asthma in children and adults.[6]

Murine models have been extensively used in studies of allergic diseases, such as food[13] and inhalant allergies.[14-16,40,42] The reasons that murine models have been actively used in the research of food and inhalant allergies are that immunologic substances used in murine models have been rapidly developed and that it is easy to manipulate mice in terms of breeding and handling. However, there are obstacles to overcome in establishing murine models, especially of food allergies. One problem is that there is a tendency that mice develop oral tolerance to antigens administered intragastrically.[39] Therefore, previously established murine models of food allergy utilized parenteral challenge and therefore did not adequately mimic human food allergy in real life.[17] In an effort to overcome the strong innate tendency of oral tolerance in mice, it became known that the age[18-20] and strain[21,22] of mice, coadministration of cholera toxin (CT),[23-26] and the nature and dose of the sensitizing protein[17,21,27,28] are important. Recently, Li *et al.* successfully established a murine model of IgE-mediated cow's milk hypersensitivity that mimics the clinical features

of immediate cow's milk hypersensitivity in humans by intragastric administration of cow's milk plus CT and boosting 5 times at weekly intervals.[13]

In the current study we used several strategies to overcome oral tolerance and induced IgE-mediated buckwheat allergy. We chose 4-week-old C3H/HeJ mice and fed them either homogenized BW (1 mg/dose) in the presence of CT as an adjuvant or CT alone. In addition, mice were sensitized intraperitoneally with HDM mixed with $Al(OH)_3$, followed by 4 times of intranasal sensitizations. Serum BW- and HDM-specific IgE antibodies were quantified by ELISA. Furthermore, the role that T cells play in the regulation of BW and HDM allergies was explored by measuring cytokine production by spleen cells from mice allergic to BW and/or HDM.

II. MATERIALS AND METHODS

1. Mice and reagents

Female C3H/HeJ mice, 4 weeks of age, were purchased from the SLC Japan (Hamamatsu, Japan) and maintained on regular mouse chow (buckwheat-free chow) under specific pathogen-free conditions. Guidelines for the care and use of the animals were followed.[29]

Freshly BW crude extract and HDM crude extract were used as antigens. Crude BW was obtained from the Korean Rural Development Administration. Crude HDM was obtained from the Department of Parasitology, Yonsei University College of Medicine. Crude BW and crude HDM extracts were prepared as follows. Cholera toxin (CT) was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA) and concanavalin A (Con A) from Sigma (St. Louis, MO, USA). Antibodies for ELISAs were purchased from PharMingen (San Diego, CA, USA). Anti-DNP IgE was purchased from Accurate Scientific Inc. (Westbury, NY, USA).

2. Buckwheat crude extract preparation

Freshly ground whole BW and crude BW extract were prepared as previously described[30] and employed as antigens. Briefly, 50 g of BW flour was defatted with

ethyl ether, and then extracted in 500 mL of phosphate-buffered saline (137 mM NaCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 27 mM KCl, pH 7.4) for 24 h at 4℃ under constant stirring. The extract was centrifuged at 10,000 g for 1 h at 4℃, and the supernatant was dialyzed (the cut-off molecular weight was 3.5 kDa; Spectrum, Houston, TX, USA) against distilled water for 48 h. The dialyzed supernatant was lyophilized and stored at -20℃ until use.

3. House dust mite crude extract preparation

Freshly ground HDM and crude HDM extract were obtained from the Department of Parasitology, Yonsei University College of Medicine, Korea, prepared as previously described[30] and employed as antigens. Briefly, 10 g of HDM was defatted with ethyl ether, and then extracted in 500 mL of phosphate-buffered saline (137 mM NaCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 27 mM KCl, pH 7.4) for 72 h at 4℃ under constant stirring. The extract was centrifuged at 50,000 g for 1 h at 4℃, and the supernatant was dialyzed (the cut-off molecular weight was 1 kDa; Spectrum, Houston, TX, USA) against distilled water for 48 h. The dialyzed supernatant was lyophilized and stored at -20℃ until use.

4. Sensitizations by intragastric administration of BW along with intraperitoneal and inhalant administrations of HDM

A. Sensitization by intragastric administration of BW

Mice were sensitized intragastrically with BW plus CT as an adjuvant on days 0, 1, 2, 7 and 18 (group 1, n=4). Two hours before intragastric sensitization was done, mouse chow was removed. Intragastric feeding was performed by means of a stainless steel blunt feeding needle. To establish the optimum sensitizing dose, mice were given 1 mg (low dose) of BW together with 10 μg/mouse of CT. The preliminary studies revealed that 1 mg/mouse of BW plus 10 μg/mouse of CT is most effective in provoking increases in the levels of IgE and IL-4 (data not shown). The BW/CT mixtures were administered in PBS at a final volume of 200 μL/mouse. Control mice received CT alone (group 2, n=4) or were left untreated (group 3, n=4).

B. Sensitizations by intraperitoneal and inhalant administrations of HDM

Mice in group 1 and 2 were sensitized with 200 μg/dose of crude HDM extract plus 400 μg/dose of $Al(OH)_3$ as an adjuvant through intraperitoneal routes (on day 0). Two weeks later (on days 14, 15, 16, and 21), mice in group 1 and 2 were given 100 μg intranasal boost of crude HDM extract under Ketamine anaesthesia. Blood samples were taken following exsanguinations on day 35. Figure 1 illustrates an overview of the study groups.

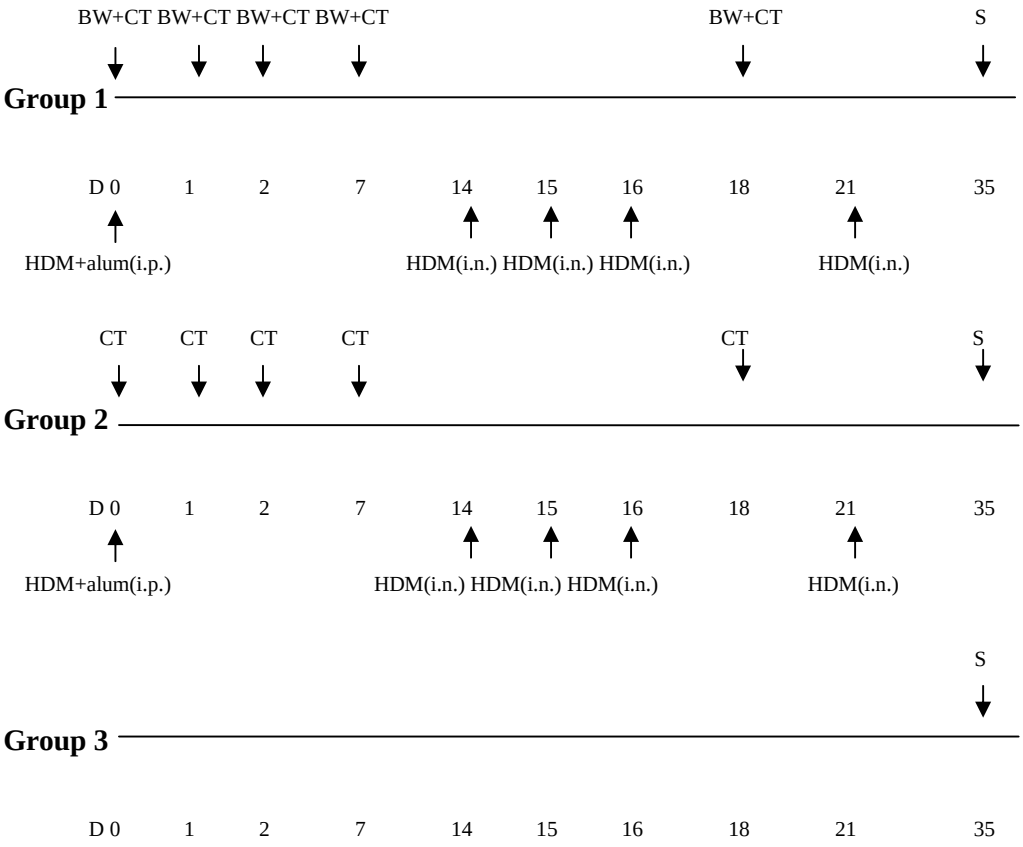

Fig. 1. Sensitization and boost protocol for murine model of buckwheat and house dust mite (each group, n=4).

BW: buckwheat, CT: cholera toxin, HDM: house dust mite, S: sacrifice

i.p.: intraperitoneal sensitization, i.n.: intranasal sensitization

5. Measurement of BW- and HDM-specific IgE, IgG1, and IgG2a in sera

Blood was obtained weekly from the tail veins of the mice during the sensitization period and was taken following exsanguinations on day 35. Sera were collected and stored at -20℃. Levels of BW- and HDM-specific IgE were measured by ELISA as previously described.[14] For measurement of BW- and HDM-specific IgG1 and IgG2a, Maxisorp Immuno 96-well plates (Nunc, Denmark) were coated with either 2 μg/mL crude BW extract or HDM extract in coating buffer (pH 9.6, Sigma, USA). After overnight incubation at 4℃, plates were washed 3 times with PBS/0.05% Tween 20 and blocked with 1% BSA-PBS for 2 hours at 37℃. After washing 3 times, serum samples (1:10 dilutions for IgE, 1:500 dilution for IgG1 and IgG2a) were added to the plates and incubated overnight at 4℃. Plates were then washed, and 100 μL of secondary antibody (anti-mouse IgE, IgG1, and IgG2a) conjugated biotin (0.5 μg/mL) were added for an additional 1 hour at room temperature (RT). After washing, reactions were added to avidin peroxidase (PharMingen, San Diego, CA, USA) for 15 minutes at RT. After 6 washings, reactions were developed with TMB substrate (PharMingen, San Diego, CA, USA) for 30 minutes at RT, and stopped with the addition of 2 N H_2SO_4, and read at 450 nm. The levels of antigen-specific IgE, IgG1, and IgG2a were calculated by comparison with a reference curve generated by using mouse mAbs (anti-DNP IgE, IgG1, and IgG2a), as previously described.[14] All analyses were performed in duplicate.

6. Comparison of quantification of cytokine proteins from splenocytes stimulated *in vitro* with BW, HDM, Con A or media

Mice were sacrificed on day 35. After spleens were removed from mice, splenocytes were ground into splenocytes using sterile 2 slides. Cells were isolated and suspended in complete culture medium (RPMI-1640 plus 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine). After 2 washings, cell numbers were counted and cell suspensions were aliquoted in 24-well flat bottom culture plates ($4x10^6$/well/mL). Cell suspensions were cultured in 24-well plates ($4x10^6$/well/mL) in the presence of BW (50 μg/mL), HDM (50 μg/mL), Con A (2 μg/mL), or media. Supernatants were collected after 72 hours of culture and stored at -20℃ for experiment. Levels of IL-4, IL-5, IL-10, IL-12, and IFN-γ were determined by ELISA, according to the manufacturer's instructions (PharMingen, CA, USA) and as previously described.[14,15] All analyses were performed in duplicate.

7. Proliferation assays

Splenocytes were isolated from pooled spleens removed from each group of mice sacrificed at week 5 and cultured in RPMI 1640 containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine. A quantity of 1 x 10^6 cells per well in 0.2 mL medium was incubated in triplicate cultures in 96-microwell plates in the presence of crude BW extract (50 μg/mL) or HDM extract (50 μg/mL). Cells

stimulated with Con A (2 μg/mL) were used as positive controls. Cells stimulated only with media were used as negative controls. Two days later, the cultures received an 18-h pulse of 1 μCi [^{3}H] thymidine per well. The cells were then harvested and the incorporated radioactivity counted in a β-scintillation counter. The results were expressed as stimulation index.

8. Statistical analysis

Statistical significance ($P < .05$) was determined by Kruskall-Wallis test or Mann-Whitney U test (rank-sum test). All statistical analyses were performed with SPSS (Chicago, IL , USA).

III. RESULTS

1. BW-specific IgE responses after intragastric BW sensitization

To determine the kinetics of IgE production in the development of BW allergy, sera from each group of mice were obtained weekly after intragastric sensitization of BW/CT, CT, or none. BW-specific IgE antibody levels were increased from week 2 through week 5, peaking at week 4 in mice sensitized with BW/CT (group 1, 98.45 ± 64.37 ng/mL) compared with the other groups not sensitized with BW (Fig 2. A), although the differences between groups were not statistically significant due to the small number of subjects. There were no significant increases in the BW-specific IgE levels in groups not sensitized with BW/CT as expected. In a preliminary study we found that sensitizing doses of 10 mg of buckwheat per mouse failed to induce BW-specific IgE response at any time point between week 1 and week 5 after sensitization (data not shown).

2. HDM-specific IgE responses after intraperitoneal and intranasal HDM sensitization

HDM-specific IgE concentrations increased significantly from week 3 through week 5, peaking at week 3 in mice sensitized with BW/CT and HDM/alum (group 1)

and also in mice sensitized with CT and HDM/alum (group 2) (169.86 ± 55.54 ng/mL, 810.52 ± 233.29 ng/mL, respectively) (Fig 2. B). Interestingly, HDM-specific IgE concentrations in mice sensitized with CT and HDM/alum (group 2) were higher than those in mice sensitized with BW/CT and HDM/alum (group 1). Repeated administrations of CT and HDM/alum induced significantly higher HDM-specific IgE levels. This response was significantly inhibited (79%) by coadministering BW intragastrically. These observations thus indicate that coimmunization with unrelated antigens, which are BW and HDM in this case, given by different routes exert a significant down-regulatory effect reciprocally.

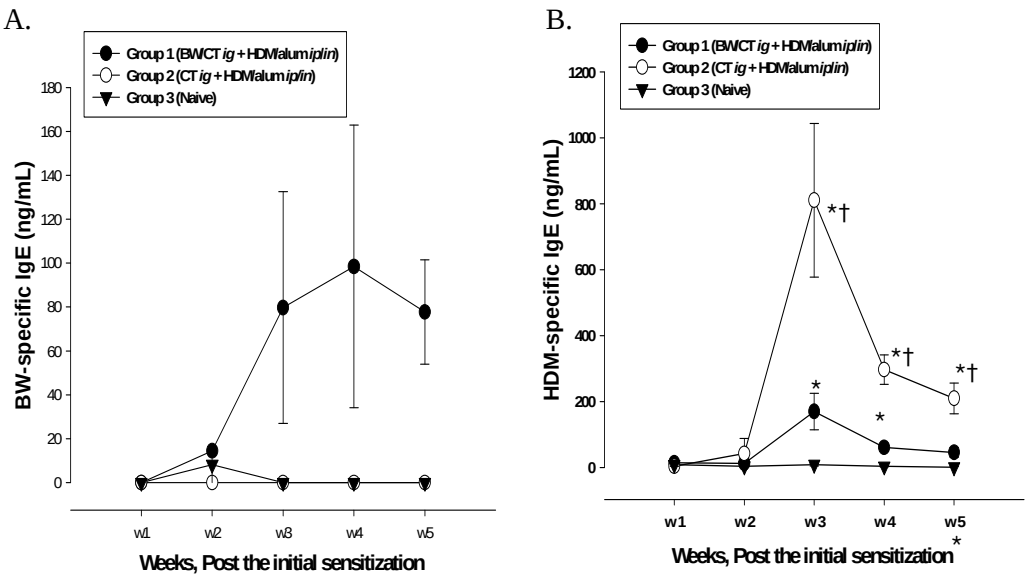

Fig. 2. Serum levels of buckwheat-specific IgE (A) and house dust mite-specific IgE (B). Sera from different groups of mice (n=4) as indicated were obtained weekly after buckwheat/cholera toxin sensitization and/or house dust mite/alum sensitization. Buckwheat- and house dust mite-specific IgE levels in pooled sera from each group were determined by ELISA. Values are expressed as mean of ± SE (* : $P < 0.05$ compared with group 3, † : $P < 0.05$ compared with group 1).

3. Increased Th2-type cytokine responses

To determine the role of T cells and cytokines in mice allergic to BW and/or HDM, we examined the production of cytokines by spleen cells from these mice. The splenocytes were stimulated *in vitro* with BW, HDM, Con A or media. It has been

suggested that IL-4 or the balance of IL-4 and IFN-γ plays a key role in regulating the plasticity of both Th1 and Th2 lineage cells and that this balance is likely to be crucial in regulation of the immune response in vivo.[31] After 72 hours in HDM-stimulated culture, IL-4 levels were increased in groups 1 and 2 mice (437.16 ± 7.06 pg/mL, 327.79 ± 9.00 pg/mL, respectively) compared with the naïve group (group 3, 37.09 ± 2.23 pg/mL) (Fig 3. A). After 72 hours in HDM-stimulated culture, IFN-γ levels were increased in groups 1 and 2 mice (593.09 ± 60.27 pg/mL, 304.65 ± 17.65 pg/mL, respectively) compared with the naïve group (group 3, 59.45 ± 12.61 pg/mL) (Fig 3. B). However, the differences in IL-4 and IFN-γ levels in HDM-stimulated culture between groups 1 and 2 were not statistically significant due to the small number of subjects.

The IL-4/IFN-γ ratio for HDM-stimulated cultures in group 2 was 1.08, the ratio being slightly higher than those in groups 1 and 3, which implies that CT and HDM/alum-sensitized mice (group 2) were more skewed toward Th2 response than BW/CT- and HDM/alum-sensitized mice (group 1) or naïve mice (group 3). When we normalized IL-4 and IFN-γ levels obtained from spleen cells stimulated *in vitro* with HDM to those levels obtained from spleen cells stimulated *in vitro* with media only, the IL-4/IFN-γ ratios for groups 1, 2 and 3 were 1.1, 1.53, and 0.93, respectively, demonstrating that the IL-4/IFN-γ ratio for CT and HDM/alum-sensitized mice (group 2) is the highest and that this response is Th2-biased (Table 1). This Th2-biased response in CT- and HDM/alum-sensitized mice (group 2) is also highly

correlated with its high HDM-IgE levels (810.52 ± 233.29 ng/mL). However IL-5, IL-10, and IL-13 levels in BW-stimulated, HDM-stimulated and unstimulated spleen cells from groups 1, 2, and 3 showed no significant differences (data not shown).

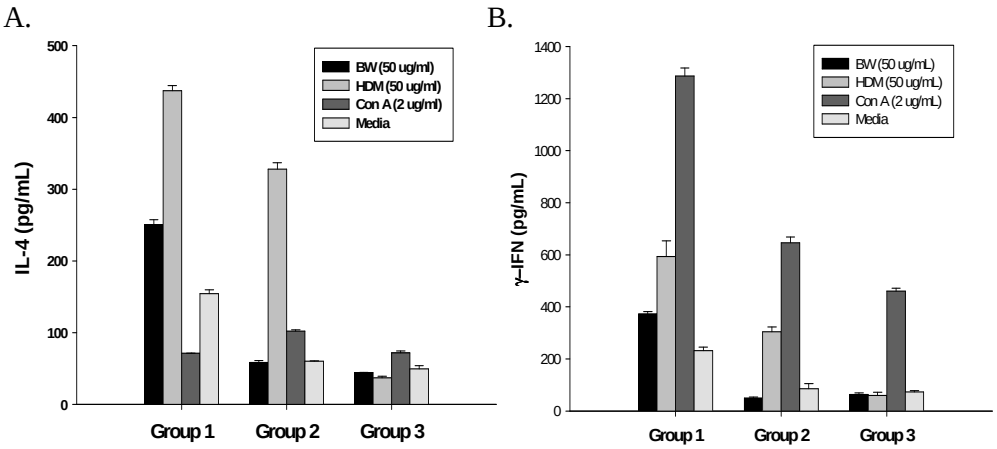

Fig. 3. Levels of IL-4 (A) and IFN-γ (B) in 72h spleen cell culture supernatants following stimulation with buckwheat, house dust mite, concanavalin A, or media (n=4). Data are given as mean ± SE.

Table 1. HDM-specific IgE levels from groups 1, 2, and 3 (n=4) and comparison of IL-4 and IFN-γ from splenocytes stimulated *in vitro* with house dust mite or media.

	IL-4*				IFN-γ*				IgE (ng/mL)	
	BW50	HDM50	Con A	Med	BW50	HDM50	Con A	Med	BW-IgE†	HDM-IgE‡
Group 1	1.62	2.83	0.46	1	1.61	2.56	5.55	1	98.45 ± 64.37	169.86 ± 55.54
Group 2	0.97	5.44	1.70	1	0.58	3.56	7.54	1	0.00 ± 0.00	810.52 ± 233.29
Group 3	0.88	0.75	1.44	1	0.85	0.81	6.26	1	0.00 ± 0.00	8.74 ± 3.95

The IL-4/IFN-γ ratios for group 1, 2 and 3 were 1.1, 1.53, and 0.93, respectively, demonstrating that the IL-4/IFN-γ ratio for CT and HDM/alum-sensitized mice (group 2) is the highest and that this response is Th2-biased. This Th2-skewed response in CT- and HDM/alum-sensitized mice (group 2) is also highly correlated with its high HDM-IgE levels (810.52 ± 233.29 ng/mL).

* : The values for IL-4 and IFN-γ were obtained by normalizing IL-4 and IFN- γ levels acquired from spleen cells stimulated *in vitro* with BW, HDM, or Con A to those levels acquired from spleen cells stimulated *in vitro* with media only.

† : The BW-specific IgE levels for each group, as stated here, were the highest values acquired at week 4 after BW/CT sensitization and/or HDM/alum sensitization. Values for IgE are expressed as mean ± SE.

‡ : The HDM-specific IgE levels for each group, as stated here, were the highest values acquired at week 3 after BW/CT sensitization and/or HDM/alum sensitization. Values for IgE are expressed as mean ± SE.

BW50 : buckwheat 50 μg/mL, HDM50 : house dust mite 50 μg/mL, Con A : concanavalin A 2 μg/mL

4. BW- and HDM-specific IgG1 and IgG2a levels and IgG1/IgG2a ratios

We also measured BW- and HDM-specific IgG1 and IgG2a levels in mice sensitized with BW and/or HDM. BW-specific IgG1 levels increased from week 2 and peaked at week 5 in mice sensitized with BW/CT and HDM/alum (8406.5 ± 444.9 ng/mL) (group 1). BW-specific IgG1 levels showed little increase in CT- and HDM/alum-sensitized mice (group 2) and in naïve mice (group 3) as expected (Fig. 4. A). HDM-specific IgG1 levels increased from week 2 and peaked at week 5 in mice sensitized with BW/CT and HDM/alum (40722.4 ± 9887.5 ng/mL) (group 1) and also at week 5 in mice sensitized with CT and HDM/alum (143360.5 ± 41858.5 ng/mL) (group 2). HDM-specific IgG1 levels showed no increase in the naïve group (group 3) (Fig. 5. A).

BW-specific IgG2a levels increased abruptly at week 5 in mice sensitized with BW/CT and HDM/alum (1015.2 ± 6.3 ng/mL) (group 1). BW-specific IgG2a levels showed no increase in mice sensitized with CT and HDM/alum (group 2) and in the

naïve group (group 3) as expected (Fig. 4. B). HDM-specific IgG2a levels increased from week 3 and peaked at week 5 in mice sensitized with BW/CT and HDM/alum (110969.0 ± 2812.0 ng/mL) (group 1) and also at week 5 in mice sensitized with CT and HDM/alum (98202.2 ± 5134.8 ng/mL) (group 2). HDM-specific IgG2a levels showed no increase in the naïve group (group 3) (Fig. 5. B).

The ratios of HDM-specific IgG1 to HDM-specific IgG2a for groups 1 and 2 at week 5 are 0.37 and 1.46, respectively, which means that CT- and HDM/alum-sensitized mice (group 2) are more Th2-deviated than BW/CT- and HDM/alum-sensitized mice (group 1). This Th2-deviated response in group 2 is also highly correlated with its high HDM-specific IgE levels (810.5 ± 233.3 ng/mL) (Table 2 and 3).

A. B.

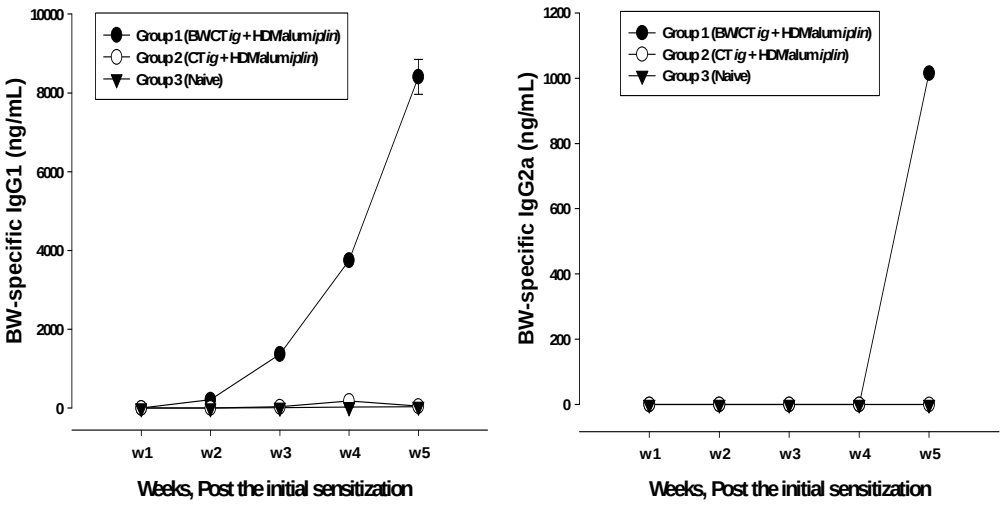

Fig. 4. Serum levels of BW-specific IgG1 (A) and IgG2a (B). Sera from different groups of mice (n=4) as indicated were obtained weekly after buckwheat/cholera toxin sensitization and/or house dust mite/alum sensitization. Buckwheat-specific IgG1 and IgG2a levels in pooled sera from each group were determined by ELISA. Values are expressed as mean ± SE (* : $P < 0.05$) (n=4).

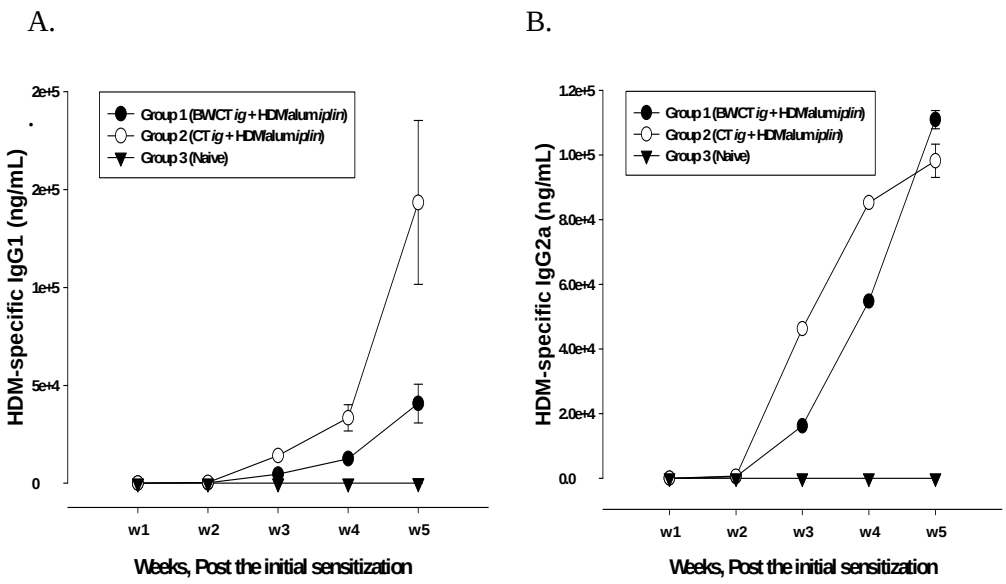

Fig. 5. Serum levels of HDM-specific IgG1 (A) and IgG2a (B). Sera from different groups of mice (n=4) as indicated were obtained weekly after buckwheat/cholera toxin sensitization and/or house dust mite/alum sensitization. Buckwheat-specific IgG1 and IgG2a levels in pooled sera from each group were determined by ELISA. Values are expressed as mean ± SE (* : $P < 0.05$) (n=4).

Table 2. Levels of buckwheat-specific IgG1 and IgG2a according to weeks after the initial sensitization (n=4).

	w 1 (IgG1/IgG2a)	w 2 (IgG1/IgG2a)	w 3 (IgG1/IgG2a)	w 4 (IgG1/IgG2a)	w 5 (IgG1/IgG2a)
Group1	0.0 ± 0.0 / 0.0 ± 0.0	210.8 ± 3.0 / 0.0 ± 0.0	1370.1 ± 22.4 / 0.0 ± 0.0	3751.3 ± 0.0 / 0.0 ± 0.0	8406.5 ± 444.9 / 1015.2 ± 6.3
Group2	0.0 ± 0.0 / 0.0 ± 0.0	0.0 ± 0.0 / 0.0 ± 0.0	28.7 ± 2.5 / 0.0 ± 0.0	177.4 ± 69.3 / 0.0 ± 0.0	46.0 ± 6.9 / 0.0 ± 0.0
Group3	0.0 ± 0.0 / 0.0 ± 0.0	0.0 ± 0.0 / 0.0 ± 0.0	8.7 ± 8.7 / 0.0 ± 0.0	24.4 ± 5.1 / 0.0 ± 0.0	36.0 ± 0.0 / 0.0 ± 0.0

Sera were pooled from each group. Serum BW-specific IgG1 and IgG2a levels were determined by ELISA (ng/mL). Values are expressed as mean ± SE.

Table 3. Levels of house dust mite-specific IgG1 and IgG2a according to weeks after the initial sensitization (n=4).

	w 1 (IgG1/IgG2a)	w 2 (IgG1/IgG2a)	w 3 (IgG1/IgG2a)	w 4 (IgG1/IgG2a)	w 5 (IgG1/IgG2a)
Group1	0.0 ± 0.0 / 0.0 ± 0.0	119.0 ± 0.8 / 388.2 ± 36.8	4617.8 ± 27.7 / 16166.4 ± 141.4	12568.1 ± 1152.9 / 54744.6 ± 433.3	40722.4 ± 9887.5 / 110969.0 ± 2812.0
Group2	14.0 ± 14.0 / 0.0 ± 0.0	377.2 ± 320.9 / 608.3 ± 3.1	14069.5 ± 654.1 / 46257.3 ± 86.5	33350.2 ± 6689.7 / 85262.3 ± 137.7	143360.5 ± 41858.5 / 98202.2 ± 5134.8
Group3	0.0 ± 0.0 / 0.0 ± 0.0	0.0 ± 0.0 / 0.0 ± 0.0	0.0 ± 0.0 / 0.0 ± 0.0	0.0 ± 0.0 / 0.0 ± 0.0	2.6 ± 2.6 / 0.0 ± 0.0

Sera were pooled from each group. Serum HDM-specific IgG1 and IgG2a levels were determined by ELISA (ng/mL). Values are expressed as mean ± SE.

5. Proliferation assays

To determine whether BW or HDM sensitization affected T cell activation, we assessed T cell proliferative responses to BW, HDM, or Con A *in vitro*. No significant differences were observed among splenocytes from all groups (data not shown).

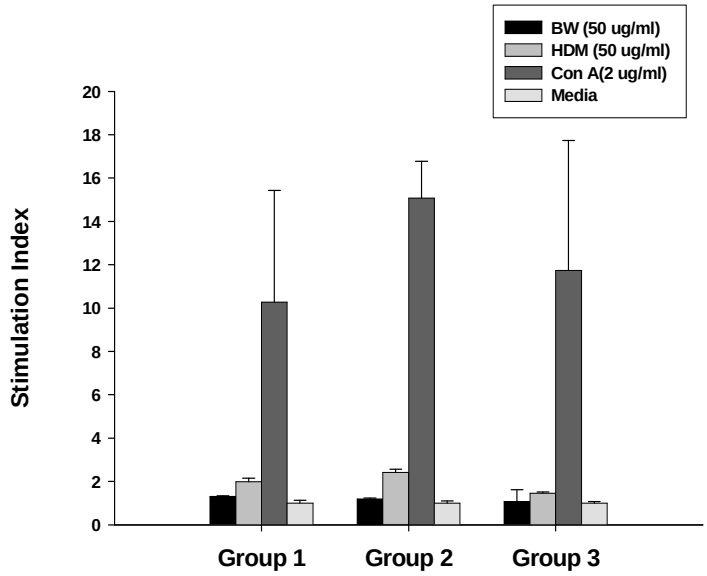

Fig. 6. Splenocyte proliferation assay after stimulation with buckwheat, house dust mite, concanavalin A, or media (48h Pulse). Splenocytes from mice in each group were stimulated with crude buckwheat or house dust mite or concanavalin A. Cells cultured in media only served as negative controls. Forty eight hours later, the cultures received an 18-h pulse of 1 μCi per well of [^{3}H] thymidine. The cells were then harvested and the incorporated radioactivity was measured. The results are expressed as a stimulation index. Data are given as mean ± SE.

IV. DISCUSSION

The prevalence of atopic disorders such as food allergy, atopic dermatitis, allergic rhinitis, and asthma has been rising in recent years, particularly in Westernized metropolitan areas. Atopic children who have atopic dermatitis or food allergy in early life are more likely to develop asthma or allergic rhinitis later in life, a phenomenon known as allergic march. In this context, there have been several studies reporting that early sensitization to food allergens in infancy should be regarded as a risk factor for the development of asthma in later years.[9,10,32,33] Studies by Sigurs *et al.* demonstrated that in 26 of 46 children (57%) who were sensitized to egg white, IgE antibodies to inhalants developed within the next 2 years, and in 19 of 25 (76%) IgE antibodies to inhalants developed before or at 12 to 15 years.[9] Thus, they concluded that sensitization to foods in infants is usually associated with appearance of IgE antibodies to inhalants later in life.[9] In a birth cohort study Rhodes *et al.* also reported that those children with positive skin prick tests to egg/milk in infancy became sensitized to aeroallergens at an earlier age when compared with those who had not shown egg/milk sensitivity.[34] Similarly, Nickel *et al.* demonstrated in a prospective, nonintervention cohort study that hen's egg–specific IgE at the age of 12 months is a valuable marker for subsequent allergic sensitization to allergens that cause asthma, allergic rhinitis, and atopic dermatitis.[10] However there have been no murine model studies in which the pathogenesis underlying the association between sensitization to

food allergens and sensitization to aeroallergens is elucidated. Previously published animal models of food allergy utilized parenteral challenge and therefore did not adequately mimic human food allergy in real life.[17] Recently, one study by Li *et al.* established a murine model of food allergy by oral sensitization and challenge, utilizing several factors.[13] On the basis of previous studies that emphasized the importance of age[18-20] and strain[21,22] of mice, coadministration of CT,[23-26] and the nature and dose of the sensitizing protein[17,21,27,28] in overcoming the strong innate tendency of oral tolerance to develop in mice, we added these well-established factors in our experimental design. We chose 4-week-old C3H/HeJ mice and fed them either homogenized BW (1 mg/dose) in the presence of CT as an adjuvant or CT alone. At the same time, the mice were sensitized with HDM (200 μg/dose) plus alum through intraperitoneal routes, followed by intranasal boost of HDM (100 μg/dose) two weeks later. Previously reported murine models focused on either food allergy or inhalant allergy and therefore did not adequately mimic what happens in human real life, where sensitizations to food and inhalant allergens occur almost simultaneously. To our knowledge, this is the first murine model demonstrating the combined sensitization of both food allergy and inhalant allergy, generated by intragastric sensitization and intraperitoneal and intranasal sensitizations, respectively.

It has been demonstrated that IgE antibodies play an important role in mediating type I hypersensitivity responses in humans.[35,36] In both food and inhalant allergies it is accepted that food- or HDM-specific IgE antibodies bind to high-affinity Fcε RI

receptors on mast cells, basophils, macrophages, and dendritic cells, as well as to low-affinity Fcε RII receptors on macrophages, monocytes, lymphocytes, eosinophils, and platelets.[37] When food or HDM allergens penetrate mucosal barriers of gastrointestinal or respiratory tract and contact IgE antibodies bound to mast cells or basophils, histamine and other mediators that induce symptoms of immediate hypersensitivity are released.[13]

Von Garnier *et al.* reported in a murine model experiment that when two unrelated antigens, phospholipase A_2 and ovalbumin, were coadministered in different combinations of doses, they may exert inhibitory effect on their reciprocal B cell response.[38] They also showed that repeated administrations of low phospholipase A_2 doses alone induced a high phospholipase A_2-specific IgE level.[38] This specific response was partially inhibited (36%) by coadministering a low ovalbumin dose, and was significantly suppressed (77%) by coimmunization with a high ovalbumin dose.[38] A similar down-regulation of the specific IgE response to ovalbumin was observed in mice coinjected with low (52%) or high phospholipase A_2 doses (70%).[38] Generally, the high equimolar dose of ovalbumin extinguished the phospholipase A_2 response.[38] Conversely the high phospholipase A_2 dose partially, but significantly down-regulated the ovalbumin response.[38] These observations thus indicated that though the dose of a given allergen may profoundly affect the polarization of the immune response, coimmunization with an unrelated antigen may exert a significant non-specific bystander effect on this response.[38] This non-specific bystander effect is

considered to be ‘negative’ because the unrelated antigens had down-regulatory effects on each other’s IgE responses. In contrast to this ‘negative’ bystander effect, Kullberg and colleagues reported that ‘positive’ bystander effects occur in murine systems, such that ongoing Th2-dominated immune responses to one antigen enhance Th2 cytokine production in response to other antigens that do not ordinarily induce Th2 cytokines.[45,46] In our experiment where repeated administrations of low doses of HDM alone (group 2) was introduced, a high HDM-specific IgE level was observed, which was also found to be highly correlated with IL-4/IFN-γ ratio (group 1 vs. group 2: 1.1 vs. 1.53) and relatively high HDM-specific IgG1/IgG2a ratio (group 1 vs. group 2: 0.37 vs. 1.46). This specific response was significantly inhibited (79%) by coadministering low doses of BW. Generally, the low equimolar dose of HDM terminated the BW response. These observations consequently indicate that cosensitization with an unrelated antigen may exert a significant non-specific ‘negative’ bystander effect on the immune response, regardless of the routes by which they are administered.

This study is different from the previous studies by von Garnier *et al.* and by Kullberg in that the two antigens used in the current study, BW and HDM, were administered by methods very similar to the actual routes by which humans become sensitized to the antigens in real life, therefore adequately mimicking human food and inhalant allergies. Thus our study demonstrates that the same pathogenesis might be responsible for the production of BW- and HDM-specific IgE in humans.

One more factor to consider is the endotoxin effect on T cell cytokine production. Endotoxin is known to augment an allergic reaction if administered before or shortly after allergen sensitization and to mitigate an allergic reaction if administered at later time points after allergen sensitization.[47] Th2 cytokine levels may not have increased as much as we expected because endotoxin might have played a role in the allergic reaction. However, since we have no clear evidence indicating that endotoxin has a direct influence on the cytokine production in this study, this issue should be addressed by future studies. On the other hand, according to our separate experimental data, no additive influence of endotoxin on the proliferative capacity and cytokine productivity of splenocytes in naïve or sham control mice was found.[48]

V. CONCLUSION

In conclusion, cosensitization with food (BW) and inhalant (HDM) allergens given by different routes resulted in partial inhibition of the production of HDM-specific IgE. This response is thought to be mediated by ‘negative’ bystander effect.

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ABSTRACT (in Korean)

위장관을 통한 메밀 감작이 복강과 비점막을 통한 집먼지진드기 특이 IgE 생성에 미치는 영향

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목적: 메밀은 한국, 일본 및 유럽에서 중요한 식품알레르기의 원인 항원 중의 하나이고, 집먼지진드기는 호흡기계 알레르기 질환에서 가장 중요한 원인 항원이다. 최근의 임상 연구들은 영아기에 식품알레르기가 있었던 소아가 그렇지 않은 소아에 비하여 후에 호흡기 알레르기가 발생할 확률이 더 높다고 보고되고 있으나, 논란의 여지가 아직 많다. 이에 본 연구에서는 메밀을 경구 감작 시킨 생쥐에게 동시에 집먼지진드기를 복강/비점막을

통해 감작하는 경우 메밀의 동시 감작이 집먼지진드기 특이 IgE 생성에 어떤 영향을 미치는지 알아보았다.

대상 및 방법: 실험동물은 4 주령의 female C3H/HeJ 를 사용하였고 group 1 은 메밀과 콜레라톡신을 실험 0, 1, 2, 7, 18 일에 경구 감작을 시키면서 집먼지진드기와 alum 을 실험 0 일에 복강 내 투여하고 추후 집먼지진드기를 14, 15, 16, 21 일에 비점막을 통해 감작시켰고, group 2 는 메밀 감작은 시행하지 않으면서, 콜레라톡신 sham 감작과 동시에 집먼지진드기 감작을 시행하였으며, group 3 는 음성 대조군으로 이용하였다. 매 주 생쥐의 꼬리 정맥에서 혈액을 채취하여 보관하였다가 감작 항원 특이 IgE 농도를 측정하였다. 실험 35 일째 생쥐를 희생시켜 비장세포 배양을 시행하여 사이토카인 생성능을 비교하였다.

결과: Group 1 의 생쥐는 실험 제 4 주에 가장 높은 메밀 특이 IgE 수치를 보였고 3 주째 가장 높은 집먼지진드기 특이 IgE 수치를 나타냈다(98.45 ± 64.37 ng/mL, 169.86 ± 55.54 ng/mL). Group 2 의 경우는 메밀 특이 IgE 항체가 음성이었고, 실험 3 주에 높은 집먼지진드기 특이 IgE 항체를 나타냈다(810.52 ± 233.29 ng/mL). 집먼지진드기 특이 IgE 생성은 group 1 에 비하여 group 2 에서 의미 있게 높았으나, IL-4 와 IFN-γ 생성능은 유사한 정도를 나타내었다.

결론: 본 연구를 통하여 생쥐모델에서 위장관을 통한 메밀 감작이 동시에 시행된 경우 복강/비점막을 통한 집먼지진드기 감작에 의한 특이 IgE 생성을 감소시키는 효과가 있음을 알 수 있었다.

핵심 되는 말: 동물 모델, 메밀, 집먼지진드기, IgE, 동시 감작