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High Dose Vitamin C Supplementation Increases the Th1/Th2 Cytokine Secretion Ratio, but Decreases Eosinophilic Infiltration in Bronchoalveolar Lavage Fluid of Ovalbumin-Sensitized and Challenged Mice

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Vitamin C is traditionally regarded to be beneficial for asthma, however the benefit is still controversial. In the present study, high dose vitamin C was supplemented to ovalbumin (OVA)-sensitized and challenged mice to evaluate the effects of dietary vitamin C on allergic asthma. In this study, the experimental mice were divided into four groups, including nonsensitized control, dietary control, positive control (cured ip with dexamethasone), and high dose vitamin C supplementation (130 mg of vitamin C/kg bw/day by gavage for 5 weeks). Differential leukocyte counts, levels of inflammatory mediators, as well as type 1 T-helper lymphocytes (Th1)-type and type 2 T-helper lymphocytes (Th2)-type cytokines in the bronchoalveolar lavage fluid (BALF) were determined. The results showed that both high dose vitamin C supplementation and dexamethasone treatments significantly ($P < 0.05$) decreased eosinophilic infiltration into BALF. High dose vitamin C supplementation significantly increased the secretion ratio of interferon (IFN)-γ/interleukin (IL)-5 cytokines. This study suggests that high dose vitamin C supplementation might attenuate allergic inflammation *in vivo* via modulating the Th1/Th2 balance toward the Th1 pole during the Th2-skewed allergic airway inflammation and decreasing eosinophilic infiltration into BALF.

KEYWORDS: High dose vitamin C supplementation; eosinophilic infiltration; Th1/Th2 cytokines; bronchoalveolar lavage fluid; ovalbumin-sensitized and challenged mouse model

INTRODUCTION

Type 1 T-helper (Th1) and type 2 T-helper (Th2) lymphocytes participate in immune responses *in vivo* (*1*). In general, Th2 cells play an important role in humoral immunity and defense against extracellular pathogens (*2*) via eosinophilic infiltration, as well as immunoglobulin E (IgE) and IgG1 production *in vivo* (*3*). Th2-type cytokines include interleukin (IL)-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (*4*). Among these, IL-4 and IL-13 are implicated in isotype switching of B-cells to produce IgE. IL-5 which promotes the differentiation, recruitment and survival of eosinophils (*4*). However, Th2-skewed immune responses may cause asthma and allergy (*5*). Asthma has been recognized as a Th2-skewed allergic disease (*6*). In contrast to the Th2 cells, Th1 cells are characterized by the production of IL-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α (*2*). Th1 cells promote cell-mediated immunity to destroy intracellular pathogens (*2*), inhibit eosinophilic infiltration, IgE and IgG1 production, and strengthen IgG2a production *in vivo* (*3*). Th1-skewed immune responses are generally pro-inflammatory and may result in autoimmune and chronic inflammatory diseases, such as type 1 diabetes mellitus. Therefore, how to maintain the Th1/Th2 balance to prevent diseases is an important issue in immunology. Dietary modification via nutrient supplementations might be practicable and throw some light on this matter in the future.

Vitamin C is an essential nutrient for human beings, required as a cofactor for many enzymes and an antioxidant for sweeping away free radicals (*7*). Among nutrients, vitamin C (ascorbic acid), having an antioxidant property which protects tissues from damage due to high free radical stress *in vivo*, plays a decisive role in human health (*8*). Epidemiological studies have suggested that a diet low in vitamin C is a risk factor for asthma (*9*). It is also suggested that lower dietary intake of antioxidant vitamin may predispose one to asthma (*10*, *11*). Vitamin C supplementation might reduce coughing and wheezing in smokers, having high oxidant stress (*12*), and attenuate exercise induced bronchoconstriction in patients with asthma (*13*). However, recent clinical studies indicated that dietary vitamin C supplementation exhibited no benefit to asthmatic patients (*14*). Apparently, effects of vitamin C supplementation on asthma seem to be still a controversial issue.

Many persons suffer from asthma. We attempted to clarify the effect of vitamin C extrasupplementation on asthma in this study. It is still difficult to evaluate the effect of nutrient interventions on acute asthmatic symptoms in human model due to asthma's complicated responses in the airways and body systems. Fortunately,

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we have established a practical ovalbumin (OVA)-sensitized and challenged mouse model for evaluating the intervention with nutrients, food constituents or health foods against asthmatic inflammation (*15*−*17*). Contrary to human beings, mice can assimilate glucose into vitamin C *in vivo* (*8*). Generally, mice do not suffer from vitamin C deficiency; however the mouse model could provide another opportunity to evaluate the effect of high dose vitamin C extrasupplementation on asthmatic inflammation *in vivo*. To clarify the effects of vitamin C supplementation on asthma, high dose vitamin C, which is equal to 1000 mg/day/person (*18*, *19*), was supplemented to OVA-sensitized and challenged mice by gavage for 5 weeks. Differential leukocyte counts, levels of pro-inflammatory mediators including prostaglandin E_2, leukotriene B_4, leukotriene C_4, histamine, nitric oxide and eotaxin, and Th1 (IFN-γ)/Th2 (IL-5) cytokines, as well as pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α cytokines in the bronchoalveolar lavage fluid (BALF) from the experimental mice were determined.

MATERIALS AND METHODS

Materials, Experimental Animals and Feeds. The experimental feed was prepared according to the recommendation of the American Institute of Nutrition AIN-76. The basic composition of feed, expressed in g/100 g, contained 40 g of sucrose, 25 g of corn starch, 20 g of casein, 5 g of fiber, 3.5 g of mineral mixture, 1 g of vitamin mixture, 0.3 g of DL-methionine, 0.2 g of choline bitartrate and 5 g of corn oil. The components of AIN-76 feed were thoroughly mixed and stored at −20 °C. There is no vitamin C in the AIN-76 feed. Female BALB/c ByJNarl mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, ROC, and maintained at the Department of Food Science and Biotechnology at National Chung Hsing University College of Agriculture and Natural Resources in Taichung, Taiwan, ROC. The animal room was kept on a 12 h light and 12 h dark cycle. Constant temperature (25 ± 2 °C) and humidity were maintained. The mice were housed and kept on a chow diet (laboratory standard diet) to acclimatize for 2 weeks before feeding the AIN-76 experimental diet. After this equilibrium period, the mice were divided into four groups, including nonsensitized control (treated ip with phosphate-buffered saline (PBS)/aluminum hydroxide (AL), coded as PBS/AL), dietary control (coded as OVA/AL), positive control (cured ip with an aliquot of 0.2 mL of dexamethasone (Sigma-Aldrich Co., St. Louis, MO; 0.2 mg/kg bw) an hour before the first aerosolized OVA challenge, coded as OVA/AL-Dex.) (*20*), and high dose vitamin C supplementation group (coded as OVA/AL-Vit. C, treated with vitamin C (130 mg/kg bw/day) by tube gavage). Vitamin C (L-ascorbic acid) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The supplemented dose of vitamin C (2.6 mg/20 g bw of mouse/day = 130 mg/kg bw of mouse/day) to mice is equal to 1000 mg/day in humans according to an appropriate conversion ratio at 1:387.9 for mice (20 g) to human (70 kg) (*19*). The 1000 mg/day of vitamin C supplementation is suggested as a high dose for humans (*18*). During the study period, mice respectively received 0.3 mL extra of deionized water or vitamin C by tube gavage. Mice food intake and body weight were measured twice a week. All experimental mice were sacrificed at day 35. The animal use protocol listed in this study has been reviewed and approved by the Institutional Animal care and Use Committee (IACUC), National Chung Hsing University, Taiwan, ROC (IACUC approval No.: 95-16).

OVA-Sensitized and -Challenged Allergic Inflammation Mouse Model. To test the effects of high dose vitamin C on airway inflammation, the mice (8 weeks old) were sensitized and challenged to induce allergic airway inflammation. The inflammation mouse model was used in our previous studies (*15*, *16*). The mice (8 weeks old) were sensitized and challenged to induce allergic airway inflammation. The mouse allergic airway inflammation model was manipulated as described by Lin et al. (*17*) and slightly modified to enhance the induction of airway inflammation. In brief, the mice were sensitized using an ip dose of 0.2 mL of alum-precipitated antigen containing 8 μg of ovalbumin (OVA, albumin chicken egg grade III, Sigma-Aldrich Co., St. Louis, MO) and 2 mg of $Al(OH)_3$ to induce primary immunity after supplementation of the specified experimental diets for one week. Two booster injections of this alum-OVA mixture were given 7 and 14 days later, respectively. Nonsensitized control mice received alum-phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 0.2 μm filtered) only. One week later, the mice were then challenged by aerosolized OVA at a concentration of 5 mg of OVA per milliliter of PBS for 60 min, twice at 3 day intervals and repeated twice in 24 h. The aerosolized OVA were produced using an ultrasonic nebulizer (sw918, Shinmed, Taipei, Taiwan). Nonsensitized control mice received PBS only. Two days later, the animals were anesthetized with diethyl ether, exsanguinated using retro-orbital venous plexus puncture and immediately euthanized using CO_2 inhalation. The bronchoalveolar lavage fluid (BALF) was collected and assayed for cytokines and other inflammatory mediators.

Collection of BALF and Cellular Differential Counts. The BALF collection and differential cell counts were modified from the methods described by Ye et al. (*21*). The mice were anesthetized with diethyl ether, exsanguinated using retro-orbital venous plexus puncture and immediately euthanized using CO_2 inhalation. Using a cannula, their lungs were immediately lavaged through the trachea with 5 aliquots of 0.6 mL of Hanks balanced salt solution (HBSS) which was free of ionized calcium and magnesium (HyClone Laboratories Inc., Logan, UT). The BALF was centrifuged at 400*g* for 10 min at 4 °C. The supernatant (BALF) volume was determined, and the supernatant was stored at −80 °C for future assay. The cell pellet was resuspended in minimum essential medium (MEM, Biological Industries, Kibbutz Beit, Haemek, Israel) containing 10% bovine serum albumin (Biological Industries, Kibbutz Beit, Haemek, Israel), and the final cell density was 1×10^6 cells/mL. The total cell count was determined with a hemocytometer using the trypan blue dye exclusion method. Cytocentrifuged preparations were stained with Liu's stain for the differential cell count. Based on standard morphological criteria, a minimum of 200 cells were counted and classified as macrophages, lymphocytes, or eosinophils (*15*). The lympocytes are mostly small cells with a condensed chromatin of nucleus and a scanty cytoplasm viewed in the light microscope (400×). Eosinophils are also small cells, but they are also called polymorphonuclear leukocytes because of their oddly (irregularly) shaped nuclei. In comparison with lymphocytes and eosinophils, macrophages are large mononuclear cells. However, monocytes, which are white blood cells with a bean-shaped nucleus, are precursors of macrophages. Differential cells are easily distinguished from each other based on the standard morphological criteria (*22*).

Assay Inflammatory Mediators in BALF. *Histamine Assay*. The BALF histamine level was determined using the Histamine-ELISA kit (SPI-BIO, Paris, France). The procedure was according to the manufacturer's instructions for use.

Prostaglandin E_2 (PGE_2) Assay. The BALF PGE_2 level was determined using the competitive enzyme immunoassay method (Prostaglandin E_2 Express EIA Kit, Cayman Chemical Co., Ann Arbor, MI).

Leukotriene B_4 (LTB_4) Assay. The BALF LTB_4 level was determined using the competitive enzyme immunoassay method (Leukotriene B_4 Express EIA Kit, Cayman Chemical Co., Ann Arbor, MI).

Leukotriene C_4 (LTC_4) Assay. The BALF LTC_4 level was determined using the competitive enzyme immunoassay method (Leukotriene C_4 Express EIA Kit, Cayman Chemical Co., Ann Arbor, MI).

Nitric Oxide (NO) Assay. Aliquots of 80 μL of BALF samples and standards (0−100 μM sodium nitrite (Sigma-Aldrich Co., St. Louis, MO) dissolved in double distilled water) were pipetted into the 96 microplate wells (Nunc, Thermo Fisher Scientific, Rockford, IL). Aliquots of 160 μL of Griess reagent were then added into each well to develop the color. The Griess reagent was freshly prepared from reagents A and B at a ratio of 1:1 (reagent A, 2% (w/v) sulfanilamide (Sigma-Aldrich Co., St. Louis, MO) dissolved in 2.5% (v/v) phosphoric acid; reagent B, 0.2% (w/v) *N*-1-naphthylethylene diamide dihydrochloride (Sigma-Aldrich Co., St. Louis, MO) dissolved in 2.5% (v/v) phosphoric acid). After incubation for 10 min, the plate was read on a plate reader (ELISA reader, ASYS Hitech, GmbH, Austria) at 540 nm. Using the standard curve, the NO concentration for each unknown sample was determined.

Protein Level Assay. The BALF protein content was analyzed using the Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Sonoma, CA), according to the accompanying instructions, using a 96-well microtiter plate.

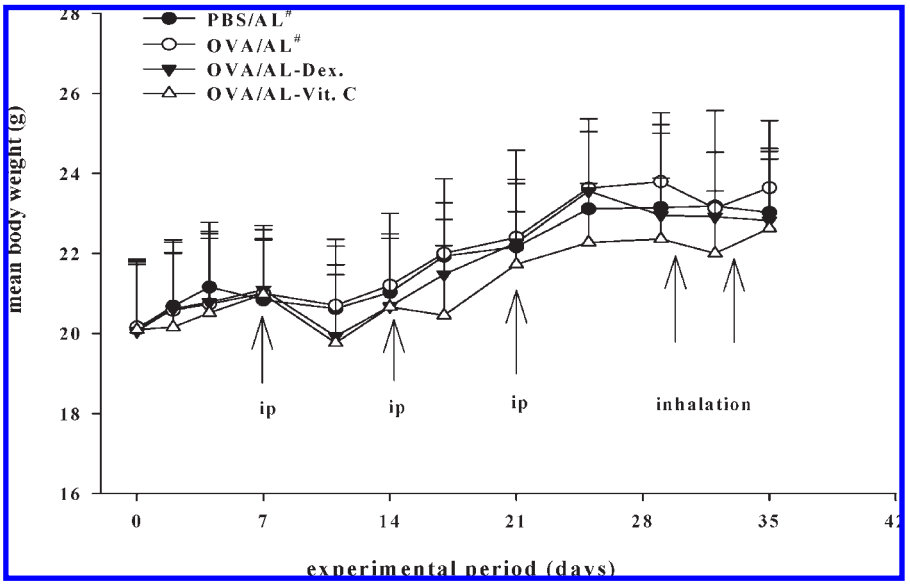

Figure 1. Effects of high dose vitamin C supplementation on body weights of OVA-sensitized and -challenged BALB/c mice. The experimental mice were sensitized with OVA/alum at days 7, 14 and 21, and then challenged twice with aerosolized OVA for 60 min twice a day at days 30 and 33. Values are mean ± SD ($n = 8-12$). There are no significant differences among groups at same experimental points. (#) Data were mentioned in our published paper (*16*). ip: Mice were intraperitoneally injected with OVA/AL or PBS/AL. Inhalation: Mice received aerosolized OVA or PBS.

Measurement of Cytokine and Chemokine Levels in BALF by an ELISA. *Determination of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ and TNF-α.* Cytokine (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, TNF-α) levels in BALF were determined using sandwich ELISA kits, respectively. The IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ and TNF-α concentrations were assayed according to the cytokine ELISA protocol of manufacturer's instructions (mouse DuoSet ELISA Development system, R&D Systems, Minneapolis, MN). The sensitivity of these cytokine assays was < 15.6 pg/mL.

Eotaxin Concentration. The BALF eotaxin concentration was determined using the mouse eotaxin sandwich ELISA kit (Quantikine M murine, R&D Systems, Minneapolis, MN). The eotaxin concentration was assayed according to the manufacturer's instructions. The sensitivity of this assay was < 15.6 pg/mL.

Statistical Analysis. Data are expressed as mean ± SD. Data within OVA-sensitized and -challenged treatments are analyzed using analysis of variance (ANOVA), followed by Duncan's test. Data between the dietary control and nonsensitized control are analyzed using unpaired Student's *t*-test. Differences among groups were considered statistically significant if $P < 0.05$. Statistical tests were performed using SPSS version 12.0.

RESULTS AND DISCUSSION

Effects of Differentially Experimental Treatments on Intake and Growth. The body weight and experimental procedure during the experimental period are given in **Figure 1**. The mean body weight of all mice slightly increased as the experimental period was extended, however there were no significant differences among groups. The feed efficiency and body weight changes of experimental mice are shown in **Table 1**. There were no significant differences in gain in body weight, feed intake, feed efficiency and body weight among the four experimental groups. There was no significant influence on intake and growth with either high dose vitamin C supplementation (OVA/AL-Vit. C) or administration with dexamethasone (OVA/AL-Dex.). The current daily recommended dietary allowance (RDA) of vitamin C is 75 mg for women and 90 mg for men (*8*). Although vitamin C appears to be relatively nontoxic (*8*), it can cause nausea and diarrhea at intake levels above 1000 mg/day for humans (*18*). To avoid possible side effects of high dose vitamin C supplementation such as nausea and diarrhea, the maximum dose of 1000 mg/day for humans, which is equal to 130 mg/kg bw/day for mice (*19*), was adopted in the present study. The present study suggests that the high dose of vitamin C supplementation we used is acceptable. Unfortunately, in this study there are no quantitative data to show what oral vitamin C supplementation has done to enrich body, plasma, and BALF ascorbate concentrations compared to the nonsupplemented group. The bioavailability of high dose vitamin C supplementation and its relative distribution in the body remain to be further clarified.

Table 1. Effects of High Dose Vitamin C Supplementation on Body Weight, Gain in Body Weight and Feed Efficiency of OVA-Sensitized and -Challenged BALB/c Mice

	groups			
items	OVA/AL[c]	OVA/AL-Dex.	OVA/AL-Vit. C	PBS/AL[c]
init body wt (g)[a,b]	20.46 ± 1.53	19.99 ± 1.77	19.85 ± 1.65	20.11 ± 1.70
final body wt (g)[a,b]	23.64 ± 0.99	22.81 ± 1.74	22.63 ± 1.73	23.02 ± 2.30
gain in body wt (g)[a,b]	3.18 ± 0.69	2.83 ± 1.06	2.78 ± 0.64	2.91 ± 0.83
intake (g/day)[a,b]	3.60 ± 0.44	3.65 ± 0.37	3.45 ± 0.39	3.57 ± 0.35
feed efficiency (%)[a,b]	2.50 ± 0.52	2.11 ± 0.46	2.21 ± 0.27	2.28 ± 0.43

[a] Values are mean ± SD ($n = 8-12$) and analyzed using analysis of variance (ANOVA), followed by Duncan's test. [b] There are no significant differences among groups within the same row. [c] Data were mentioned in our published paper (*16*).

Effects of High Dose Vitamin C Supplementation on Cellularity and Inflammatory Mediator Levels in BALF of OVA-Sensitized and -Challenged Mice. The cellularity in BALF from experimental mice is shown in **Table 2**. The OVA-sensitization and challenge treatments significantly influenced the constituents of BALF cells (OVA/AL versus PBS/AL). The OVA-sensitization and challenge treatments significantly ($P < 0.05$) decreased the percentage of monocytes/macrophages (decrease from 86.3 ± 11.7% to 32.2 ± 11.5%), but increased the percentage of eosinophils (increase from 1.9 ± 1.1% to 66.0 ± 12.4%). The OVA-sensitization and -challenge treatments also slightly ($P > 0.05$) increased total cell number, but decreased the percentage of lymphocytes (decrease from 11.7 ± 11.3% to 1.87 ± 1.69%). Administration with high dose vitamin C (130 mg/kg bw/day) to experimental mice slightly ($P > 0.05$) increased the total cell number (increase from

Table 2. Effects of High Dose Vitamin C Supplementation on Cellularity in Bronchoalveolar Lavage Fluid of OVA-Sensitized and -Challenged BALB/c Mice

	groups			
items	OVA/AL[c]	OVA/AL-Dex.	OVA/AL-Vit. C	PBS/AL[c]
total cells ($\times 10^5$/mouse)[a,b]	21.5 ± 14.4 ab	19.3 ± 20.9 ab	27.7 ± 16.5 b	5.73 ± 3.36 a
monocytes/macrophages (%)[a,b]	32.2 ± 11.5 a	53.3 ± 17.1 b	47.4 ± 20.7 ab	86.3 ± 117 c
lymphocytes (%)[a,b]	1.87 ± 1.69 a	22.3 ± 9.2 b	11.9 ± 9.5 ab	11.7 ± 11.3 ab
eosinophils (%)[a,b]	66.0 ± 12.4 c	24.4 ± 16.8 b	40.8 ± 16.9 b	1.9 ± 1.1 a

[a] Values are mean ± SD ($n = 6-9$). [b] Values within the same row not sharing common letters are significantly different ($P < 0.05$) from each other analyzed by analysis of variance (ANOVA), followed by Duncan's test. [c] Data were mentioned in our published paper (*15*).

21.5 ± 14.4 to $27.7 \pm 16.5 \times 10^5$ cells/mouse), but markedly ($P < 0.05$) decreased the eosinophilic infiltration into BALF (decrease from 66.0 ± 12.4% to 40.8 ± 16.9%), resembling the effect of dexamethasone treatment (OVA/AL-Dex.) (decrease from 66.0 ± 12.4% to 24.4 ± 16.8%). Through the dexamethasone treatment by intraperitoneal injection to mice, the percentages of monocytes/macrophages (increase from 32.2 ± 11.5% to 53.3 ± 17.1%) and lymphocytes (increase from 1.87 ± 1.69% to 22.3 ± 9.2%) significantly ($P < 0.05$) increased, but eosinophils significantly diminished (decrease from 66.0 ± 12.4% to 24.4 ± 16.8%) in BALF.

Table 3 shows the eotaxin, protein, LTB_4, LTC_4, histamine and nitric oxide (NO) levels in BALF, however there were no significant differences among the differential treatments. **Table 3** also shows that OVA-sensitization and -challenge treatments significantly influenced the PGE_2 level, however high dose vitamin C supplementation or dexamethasone treatments did not significantly affect the PGE_2 level in BALF. The results suggest that high dose vitamin C administration did not significantly affect protein, eotaxin and other inflammatory mediator levels in BALF. But, the mitigating effects of dexamethasone treatment on inflammatory mediator levels in BALF of OVA-sensitized and challenged mice also did not appear.

In this study, the results showed that OVA-sensitization and -challenge significantly increased the flux of total leukocytes, especially eosinophils, as well as PGE_2 release into the bronchoalveolar tissues (**Tables 2** and **3**), exhibiting both airway inflammation and Th2-skewed inclination in BALF. The levels of nonspecific and anti-OVA specific IgE were also significantly increased in the sera after OVA-sensitization and -challenge treatments in the established mouse model (*16*). After 5 weeks feeding, high dose vitamin C supplementation exhibited anti-allergic effects via significantly decreasing eosinophilic infiltration into the airways (**Table 2**). The decrease of eosinophilic infiltration into BALF suggested that high ascorbic acid level might remodel the inflammatory condition of the airways via decreasing vasodilatation, mucus hypersecretion and bronchoconstriction (*23*, *24*). It is also found that OVA-sensitized guinea pigs decreased in airway responsiveness after 30-day administration of ascorbic acid (*25*). Dietary vitamin C intake reduced the wheezing symptoms in childhood (*26*) and in smokers (*12*), and attenuated exercise-induced bronchoconstriction in asthmatic patients supplemented with ascorbic acid (1500 mg/day) for 2 weeks (*13*). It has been reported that vitamin C has an anti-inflammatory action in decreasing leukocyte adhesion to the endothelium (*7*). However, total leukocytes did not decrease in BALF from the mice supplemented with high dose vitamin C, although the eosinophilic infiltration significantly decreased in the present study (**Table 2**). The present study suggests that high dose vitamin C supplementation might decrease the infiltration of eosinophils into the endothelium of trachea.

Effects of High Dose Vitamin C Supplementation on Pro-Inflammatory, as Well as Th1-Type and Th2-Type Cytokine Levels in BALF of OVA-Sensitized and -Challenged Mice. Table 4 shows

Table 3. Effects of High Dose Vitamin C Supplementation on Inflammatory Mediator Levels in Bronchoalveolar Lavage Fluid of OVA-Sensitized and Challenged BALB/c Mice

	groups			
inflammatory mediators[a,b,c,d]	OVA/AL[g]	OVA/AL-Dex.	OVA/AL-Vit. C	PBS/AL[e,g]
protein (μg/mouse)	368 ± 139	392 ± 179	452 ± 140	266 ± 96
eotaxin (pg/mouse)	356 ± 107	469 ± 200	427 ± 152	270 ± 108
PGE_2[f] (ng/mouse)	6.75 ± 3.95	5.25 ± 5.42	6.05 ± 4.35	1.18 ± 0.80*
LTB_4[f] (pg/mouse)	10.13 ± 1.95	10.86 ± 5.29	13.11 ± 4.34	8.85 ± 1.39
LTC_4[f] (pg/mouse)	4.11 ± 1.70	3.90 ± 1.25	4.83 ± 2.26	4.84 ± 1.77
histamine[f] (pmol/mouse)	9.79 ± 3.55	10.11 ± 3.64	8.88 ± 1.98	10.40 ± 3.94
NO[f] (nmol/mouse)	5.68 ± 2.83	6.25 ± 2.19	5.45 ± 0.99	5.10 ± 1.57

[a] Values are mean ± SD ($n = 6-11$). [b] Data within the same row among OVA-sensitized and -challenged treatments are analyzed using analysis of variance (ANOVA), followed by Duncan's test. [c] Data within the same row between OVA/AL and PBS/AL groups are analyzed by unpaired Student's *t*-test. [d] No significant differences were found among OVA-sensitized and -challenged groups within the same row. [e] Asterisk (*) means significantly ($P < 0.05$) different between OVA/AL and PBS/AL groups. [f] The detection limits of the kits used in this study: PGE_2, 36 pg/mL; LTB_4, 13 pg/mL; LTC_4, 10 pg/mL; histamine, 0.5nM. [g] Data were mentioned in our published paper (*15*).

the levels of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 in BALF of OVA-sensitized and -challenged mice through 5 weeks feeding. The results showed that sensitization and challenge with OVA did not significantly influence the secretion of the pro-inflammatory cytokines. Contrary to our expectation, dexamethasone administration significantly ($P < 0.05$) increased these pro-inflammatory cytokine levels in BALF of OVA-sensitized and -challenged mice; IL-1β level increased from 29.6 ± 19.6 to 49.3 ± 14.7 pg/mouse, TNF-α level increased from 216 ± 128 to 367 ± 126 pg/mouse and IL-6 level increased from 257 ± 134 to 405 ± 123 pg/mouse. The results indicated that the high vitamin C supplement did not alleviate pro-inflammatory cytokines in BALF of OVA-sensitized and -challenged mice, however dexamethasone administration might increase pro-inflammatory cytokine secretions in BALF of the experimental mice.

To further evaluate the effects of high dose vitamin C supplementation on the secretion of Th1/Th2 cytokines in local tissues of the lungs and the airways, the amounts of Th1 cytokines (IFN-γ and IL-2) and Th2 cytokines (IL-4, IL-5 and IL-10) in BALF were measured (**Table 4**). The results showed that high dose vitamin C supplementation did not significantly affect the secretion levels of Th1 and Th2 cytokines in BALF. However, the levels of IFN-γ (Th1), IL-5 and IL-10 (Th2) were significantly increased in BALF from the dexamethasone-administrated mice; IFN-γ level increased from 78 ± 56 to 155 ± 76 pg/mouse, IL-5 level increased from 222 ± 110 to 373 ± 103 pg/mouse and IL-10 level increased from 418 ± 190 to 657 ± 213 pg/mouse. The secretion ratios of IFN-γ (Th1)/IL-5 (Th2) in BALF of the experimental mice are shown in **Figure 2**. The results showed that high dose vitamin C supplementation significantly increased

Table 4. Effects of High Dose Vitamin C Supplementation on Pro-Inflammatory as Well as Th1- and Th2-Type Cytokine Levels in Bronchoalveolar Lavage Fluid of OVA-Sensitized and -Challenged BALB/c Mice

	groups			
	OVA/AL[g]	OVA/AL-Dex.	OVA/AL-Vit. C	PBS/AL[g]
Pro-Inflammatory Cytokine Levels[a,b,c,d,e,f] (pg/mouse)				
IL-1β	29.6 ± 19.6 a	49.3 ± 14.7 b	29.6 ± 13.4 a	34.3 ± 26.5
TNF-α	216 ± 128 a	367 ± 126 b	243 ± 98 ab	280 ± 223
IL-6	257 ± 134 a	405 ± 123 b	270 ± 103 ab	289 ± 204
Th1-Type Cytokine Levels[a,b,c,d,e,f] (pg/mouse)				
IFN-γ	78 ± 56 a	155 ± 76 b	92 ± 45 ab	119 ± 105
IL-2	153 ± 42 a	203 ± 76 a	157 ± 58 a	142 ± 85
Th2-Type Cytokine Levels[a,b,c,d,e,f] (pg/mouse)				
	OVA/AL[g]	OVA/AL-Dex.	OVA/AL-Vit. C	PBS/AL[g]
IL-4	161 ± 84 a	196 ± 67 a	158 ± 31 a	161 ± 90
IL-5	222 ± 110 a	373 ± 103 b	262 ± 112 ab	265 ± 196
IL-10	418 ± 190 a	657 ± 213 b	470 ± 142 ab	548 ± 315

[a] Values are mean ± SD (n = 6−11). [b] Data within the same row among OVA-sensitized and -challenged treatments are analyzed using analysis of variance (ANOVA), followed by Duncan's test. [c] Data within the same row between OVA/AL and PBS/AL groups are analyzed by unpaired Student's t-test. [d] Values within the same row among OVA-sensitized and -challenged treatments not sharing common letters are significantly different (P < 0.05) from each other. [e] There are no significant differences between OVA/AL and PBS/AL groups within the same row. [f] The sensitivity of the ELISA kits used in this study was about <15.6 pg/mL. [g] Data were mentioned in our published paper (*15*).

the secretion ratio of Th1/Th2 cytokines in BALF (increased from 0.36 ± 0.06 to 0.56 ± 0.24), suggesting that high dose vitamin C supplementation modulated the Th1/Th2 balance toward the Th1 pole.

In addition to serving as an antioxidant *in vivo*, the immune effects of vitamin C have been discussed (*7*). Low levels of plasma vitamin C were found in patients with severe asthma (*27*). A case-control study indicated that plasma vitamin C and dietary vitamin C intake showed a positive significant correlation, and plasma and leukocyte vitamin C levels were significantly lower in the asthma groups (*28*). Vitamin C exhibited the effects to modulate leukocyte phagocytic action, immunoglobulin production, chemotaxis and adhesiveness in some *in vitro* studies (*29*). Duarte and Lunec (*8*) reviewed recent cultured cell studies, and addressed that vitamin C can affect gene expression, possibly through its effects on redox-sensitive signaling pathways. Lee et al. (*30*) indicated that antioxidants may reduce IL-18 (a pro-inflammatory cytokine) expression by inhibiting the activity of nuclear factor (NF)-κB in an OVA-induced asthmatic murine model. Collectively, vitamin C may have anti-inflammatory effects in asthma. However, results from our study showed that high dose vitamin C supplementation did not decrease the levels of inflammatory mediators such as prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4), histamine, nitric oxide and eotaxin (**Table 3**), and pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (**Table 4**) in BALF. Interestingly, the high dose vitamin C supplementation could significantly increase the Th1/Th2 (IFN-γ/IL-5) cytokine secretion ratio in BALF (**Figure 2**), although it did not significantly change the levels of Th1 and Th2 cytokines in BALF (**Table 4**). This study suggests that high dose vitamin C supplementation regulated the Th1/Th2 cytokine profile toward the Th1 pole in allergic asthma. We suggest that high dose vitamin C might modulate the Th1/Th2 cytokine gene expression *in vivo*. However, the immunomodulatory mechanisms of high dose vitamin C supplementation *in vivo* remain to be further clarified.

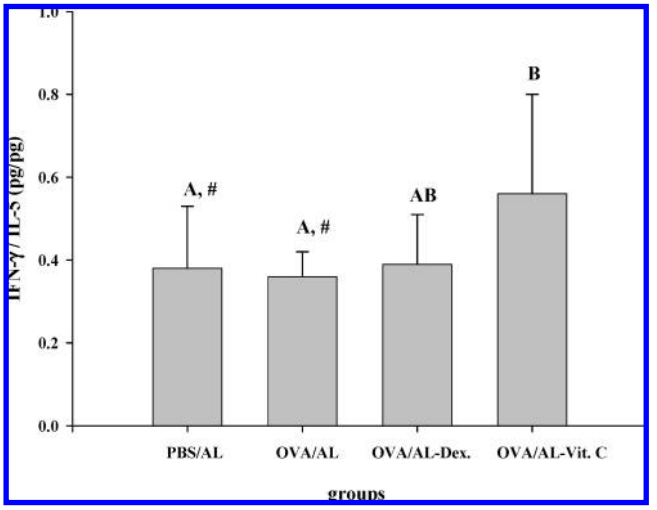

Figure 2. Effects of high dose vitamin C supplementation on the IFN-γ/IL-5 cytokine secretion ratio in bronchoalveolar lavage fluid from OVA-sensitized and -challenged BALB/c mice. Values are mean ± SD (n = 6−11). Bars not sharing common capital letters are significantly different (P < 0.05) from each other analyzed using analysis of variance (ANOVA), followed by Duncan's test. (#) Data were mentioned in our published paper (*15*).

To compare the effects of high dose vitamin C supplementation, dexamethasone, a potent synthetic member of the glucocorticoid, was selected as positive control for its anti-inflammatory and immunosuppressant activities in this study. The OVA-sensitized and -challenged mice in OVA/AL/-Dex. group were treated ip with dexamethasone an hour before the first aerosolized OVA challenge (*20*). The results showed that dexamethasone treatment effectively improved the cellularity in BALF (**Table 2**), however it also increased the secretion levels of pro-inflammatory cytokines (**Table 4**), and both Th1 and Th2 cytokines (**Table 4**). Although most Th2 cytokines, especially IL-10 (**Table 4**), might exert an anti-inflammatory activity through inhibiting Th1 and pro-inflammatory cytokine secretions (*4*, *31*), the results from this study also suggest that dexamethasone treatment for allergic asthma *in vivo* should be prudent. In contrast to the possible side effects of dexamethasone treatment, high dose dietary vitamin C might provide a moderate protection against eosinophilic infiltration into bronchoavelolar tissues (**Table 2**). Although the highly differential immune responses among individual mice resulted in no significant difference of IL-2/IL-4 (Th1/Th2) secretion in BALF, high dose vitamin C supplementation was still favorable to increase the Th1/Th2 (IFN-γ/IL-5) cytokine secretion ratio in BALF (**Figure 2**). Both eosinophil infiltration and levels of IFN-γ/IL-5 cytokines in BALF from the airways and the lungs of experimental mice were respectively confirmed using the cellular differential counts (**Table 2**) and ELISA method (**Figure 2**) in the present study. The results from this study are valuable for confirming the status of asthma and allergic inflammation. Unfortunately, the present study did not further confirm the expression of eosinophils CCR3 (chemokine (C-C motif) receptor 3) and chemokines in the lungs using in situ hybridization and immunohistochemistry (IHC) methods. The in situ hybridization and IHC methods to determine that expression of eosinophils CCR3 and chemokines in the lungs of experimental mice remain to be further studied.

In conclusion, the results from this study showed that high dose vitamin C supplementation significantly decreased eosinophilic infiltration and increased the Th1/Th2 (IFN-γ/IL-5) cytokine

secretion ratio in BALF. This study suggests that high dose vitamin C supplementation might attenuate allergic inflammation *in vivo* via modulating the Th1/Th2 balance toward the Th1 pole during the Th2-skewed allergic airway inflammation and decreasing eosinophilic infiltration into BALF.

ABBREVIATIONS USED

Vit. C, vitamin C; Th1, type 1 T-helper lymphocytes; Th2, type 2 T-helper lymphocytes; BALF, bronchoalveolar lavage fluid; IFN-γ, interferon-γ; IL-5, interleukin-5; OVA, ovalbumin; AL, aluminum hydroxide; DEX, dexamethasone.

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