

Mega-dose vitamin C attenuated lung inflammation in mouse asthma model

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Abstract: Asthma is a Th2-dependent disease mediated by IgE and Th2 cytokines, and asthmatic patients suffer from oxidative stresses from abnormal airway inflammation. Vitamin C is a micro-nutrient functioning as an antioxidant. When administered at a mega-dose, vitamin C has been reported to shift immune responses toward Th1. Thus, we tried to determine whether vitamin C exerted beneficial effects in asthma animal model. Asthma was induced in mice by sensitizing and challenging with ovalbumin. At the time of challenge, 3~5 mg of vitamin C was administered and the effects were evaluated. Vitamin C did not modulate Th1/Th2 balance in asthma model. However, it decreased airway hyperreactivity to methacholine, decreased inflammatory cell numbers in bronchoalveolar lavage fluid, and moderate reduction of perivascular and peribronchiolar inflammatory cell infiltration. These results suggest that vitamin C administered at the time of antigen challenge exerted anti-inflammatory effects. Further studies based on chronic asthma model are needed to evaluate a long-term effect of vitamin C in asthma. In conclusion, even though vitamin C did not show any Th1/Th2 shifting effects in this experiment, it still exerted moderate anti-inflammatory effects. Considering other beneficial effects and inexpensiveness of vitamin C, mega-dose usage of vitamin C could be a potential supplementary modality for the management of asthma.

Key words: Asthma, Vitamin C, Lung inflammation, Th1/Th2 balance

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Introduction

Allergic asthma is a chronic obstructive inflammatory lung disease characterized by increased reactivity of airway smooth muscles and remodeling of airway tracts. Traditionally, asthma is thought to be a Th2-dependent disease and is known to be mediated by elevated serum IgE levels (Barrios *et al.*, 2006). Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13 play major roles in the initiation and perpetuation of the disease (Barrios *et al.*, 2006; Tulic & Hamid, 2006; Doherty & Broide, 2007), although TNF- α , the Th1 cytokine, is also a critical factor in severe cases (Holgate *et al.*, 2006). The

prevalence of asthma is increasing over and over worldwide, and in addition, it is sometimes refractory to conventional treatment (Chanez *et al.*, 2007).

Because it is well established that asthmatic patients suffer from oxidative stresses from abnormal airway inflammation (Kelly *et al.*, 1999; Schock *et al.*, 2003), a lot of studies have been devoted to elucidating the relationship between dietary intake and serum level of antioxidants and the prevalence and severity of the disease (Gao *et al.*, 2008; Sackesen *et al.*, 2008). One of the most probably associated dietary antioxidants with asthma is vitamin C (Harik-Khan *et al.*, 2004).

Vitamin C is an essential micro-nutrient involved in several metabolic pathways (Naidu 2003) and is regarded as an important physiological antioxidant (Guaiquil *et al.*, 2001). Most mammals produce vitamin C by themselves, while some mammals including men, some primates, and guinea pig do not. Thus, men have to be supplemented with exogenous vitamin C by taking fresh fruits and vegetables,

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and/or supplementary pills. It is well known that the deficit of vitamin C lead to the scurvy (Naidu 2003).

Vitamin C is also known to modulate immune responses. Recently, it has been reported that mega-dose vitamin C administered in mice shifted immune responses toward Th1 with suppression of Th2 responses (Noh *et al.*, 2003; Noh *et al.*, 2005). Conversely, inadequate vitamin C supplementation in L-gulonolactone oxidase deficient mice which can not synthesize vitamin C by themselves (Maeda *et al.*, 2000) resulted in a decreased Th1 response against *H. pylori* infection (Lee *et al.*, 2008).

Considering the pathophysiology of asthma, vitamin C could be used for the treatment and/or management of asthma as an antioxidant and/or as a Th1-shifting agent. Actually, studies on the beneficial effects of high dose vitamin C intake in asthmatic patients have been performed by some authors. However, the effects are still in controversy (Fogarty *et al.*, 2003; Fogarty *et al.*, 2006; Tecklenburg *et al.*, 2007; Kaur *et al.*, 2009). This controversy might come from differences in experimental settings such as criteria for patient selection, recommended dosage of vitamin C, parameters measured, and so on.

In this study, we tried to determine the effect of vitamin C on asthma in an experimental animal model. Our results showed that vitamin C did not changed Th1/Th2 balance in asthma. However, it exerted some anti-inflammatory effects against lung inflammation.

Materials and Methods

Experimental animals

Eight week-old male Balb/c mice were purchased from BioLink (Seoul, Korea) and kept under 12 h dark and light cycle in the Animal Facility of our Institute. Food and water were given *ad libitum*. Animal experiments were permitted by the Institute of Laboratory Animal Resources Seoul National University (permission number SNU-060920-5), and all the animal experimental procedures were performed following the SOP of our Institute.

Induction of experimental asthma model

Mice were sensitized on 3 consecutive days by intraperitoneal injection of 100 µg ovalbumin (OVA; Sigma, St. Louis, MO) immersed in a same volume of alum (Peirce, Rockford, IL). On days 18, 19, 22, and 23, mice were

challenged with intranasal dripping of 25 µg OVA/30 µl PBS. On days from 18 to 24, mice were given an intraperitoneal injection of either 3 or 5 mg vitamin C dissolved in 100 µl PBS or of a same volume of PBS. Each experimental group consisted of 8 mice. On day 24, plethysmography was done. All experiments were repeated 3 times.

Airway hyperresponsiveness (AHR)

AHR was assessed indirectly by measuring methacholine-induced airway obstruction. Mice were challenged with serially increasing concentrations of nebulized methacholine (Sigma, St. Louis, MO) in a range of 0~50 mg/ml for 3 min and were placed in a whole body plethysmography chamber (Allmedicus Co., Ltd, Model # OCP-3000) after each concentration of methacholine exposure. Breathing pattern was recorded for 2 min and enhanced pause (Penh) was calculated using a software program based on measured parameters such as expiratory time, relaxation time, peak expiratory flow, and peak inspiratory flow.

Bronchoalveolar lavage and tissue processing

Mice were sacrificed on day 25 by deep anesthesia. After anesthesia, blood was drawn from orbital plexus and serum was obtained after clotting in a refrigerator. Bronchoalveolar lavage fluid (BALF) and lung tissue were obtained immediately after sacrifice. BAL was done with 1 ml of saline. Obtained fluids were centrifuged (4,000 rpm, 10 min) to get sedimented cells and supernatants. Cells were resuspended in 300 µl PBS and were used for cell count and for monolayer cell spread by cytopsin (Shandon Cytospin 2) (800 rpm, 10 min). Spread cells were stained with Wright's staining solution (Sigma, St. Louis, MO) and used for differential cell counts. Supernatants were kept frozen until use for cytokine ELISA (Biosource, Camarillo, CA).

After BAL, lungs were inflated with 1 ml Bouin's solution and further fixed in the same solution for 24 h. Routine processes for paraffin embedding were carried out, and 6 µm-thick paraffin sections were made. Tissue sections were subjected to hematoxylin and eosin staining for routine microscopic observation, and to periodic acid-Schiff's staining for goblet cell observation in the airway epithelium.

Histopathological scoring of lung tissues

The intensity of peribronchial and perivascular inflammatory cell infiltration was scored as the approximate number of layers of cells around vessels or bronchioles. In

a tissue section, the intensity of infiltration varied for each vessel and airway, and thus the most severely infiltrated one was regarded as a representative one for the section. The degree of goblet cell hyperplasia was evaluated as the ratio of respiratory epithelium composed of PAS-positive goblet cells to the total epithelium. To get the ratio, the length of all the respiratory tracts shown in the section was measured and summed with respect to normal or PAS positive area. Measurement was done using image analysis software RHINOCEROS 4.0 program.

ELISA for serum antigen-specific antibody titration

Titers of OVA-specific antibodies in the serum were determined as follows. Ninety six-well plates (Nunc, Rochester, NY) were coated with 100 μ l of 3 μ g/ml OVA/well at 4°C overnight. Plates were then briefly washed and blocked with 1% skim milk in PBS for 1 h at room temperature. One hundred-fold diluted serum samples were applied to the first row of a plate and 4-fold serial dilution was done. All samples were duplicated. After 2 hour-incubation at room temperature, plates were washed with a PBS-0.05% Tween 20 mixture, and alkaline phosphatase-conjugated antibodies for each isotype were added. After incubation for 60 min at room temperature, plates were washed and p-nitrophenyl phosphate substrate (Amresco, Solon, OH) solution was added. OD values were measured at 405 nm. The antibodies used were as follows; goat anti-mouse polyvalent immunoglobulins (1 : 1,000 diluted, Sigma), goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE antibodies (1 : 1,000 diluted, Southern Biotech, Birmingham, AL). OVA-specific anti-sera obtained from previous experiments were collected in a tube and used as a standard serum in every ELISA plate. Titers were expressed as a relative value to the standard serum value.

Statistical analysis

Statistical analysis was performed by Student's t test or Wilcoxon rank sum test. Statistical significance was set at $P < 0.05$.

Results

Vitamin C administered during challenge reduced airway resistance induced by methacholine

Airway resistance induced by methacholine inhalation was evaluated by plethysmography (Fig. 1). Airway resistances

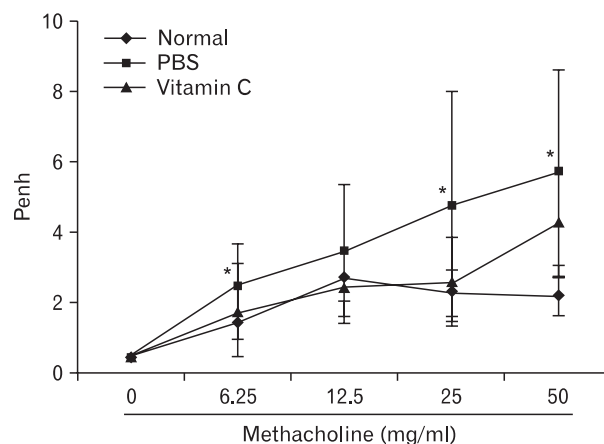


Fig. 1. Plethysmography of normal and asthma-induced mice. Mice were inhaled with aerosol containing methacholine at concentrations indicated and placed in the plethysmography chamber. Breathing was recorded for 2 min and airway resistance was calculated thereby. PBS-treated mice with asthma (■) showed increased airway resistance compared to normal mice (◆) at all concentrations of methacholine. Meanwhile, vitamin C-treated mice with asthma (▲) showed decreased resistance than that of PBS-injected mice, and that was similar to that of normal mice at methacholine concentrations from 6.25 to 25 mg/ml. * $P < 0.05$.

in PBS-injected asthma group were significantly higher with all concentrations of methacholine ($P < 0.05$) compared to those of normal control group. In contrast, vitamin C-treated asthma group showed similar resistances to those of normal control group with 6.25, 12.5, 25 mg/ml of methacholine. However, resistance with 50 mg/ml of methacholine was elevated compared to normal control group, which was still lower than that of PBS-injected asthma group. These results indicate that vitamin C administered during antigen challenge reduced AHR.

Vitamin C treatment reduced the number of inflammatory cells in bronchoalveolar lavage fluid

To approximate the extent of inflammation in each group, total numbers of inflammatory cells in bronchoalveolar lavage fluid (BALF) were counted. The numbers were standardized to the volume of fluid re-collected and depicted in Fig. 2 (left panel). In normal control group, BALF cells were scanty, while cell numbers substantially increased both in PBS-injected and vitamin C-treated asthma groups. Of note is that the number of cells from vitamin C-treated group was lower than that from PBS-injected group with statistical significance ($P < 0.05$). To investigate the composition of BALF cells, they were spread on a glass slide, stained with Wright's solution,

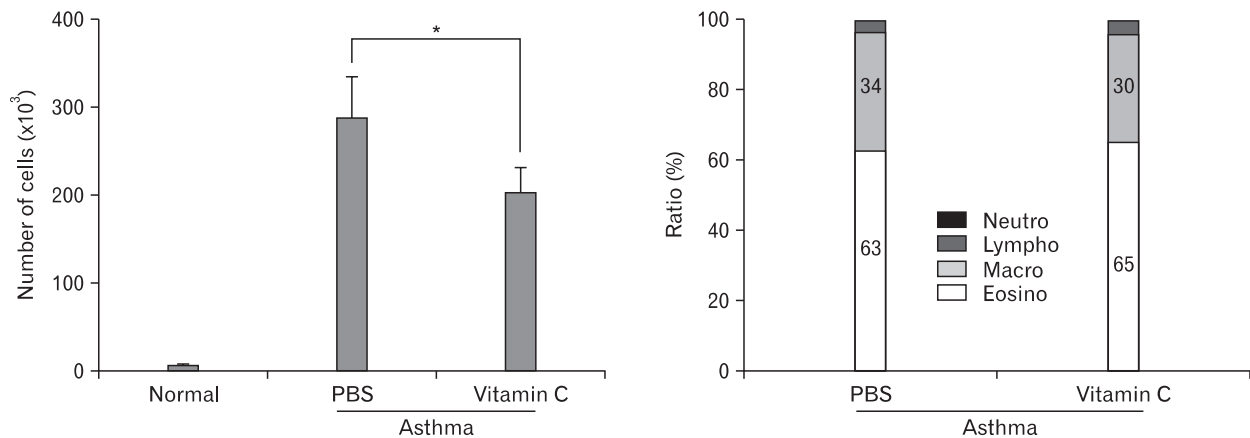


Fig. 2. (left panel) Cell numbers in BALF. Mice were anesthetized and 1 ml of saline was infused into the lung through a needle in the trachea. The fluid was re-drawn after 1 min and centrifuged. Obtained cells were re-suspended, and cell count was done. Scanty cells were found in BALF from normal mice while many inflammatory cells were observed in BALF from asthma-induced mice. Vitamin C-treated mice showed less number of BALF cells than PBS-injected mice. A representative profile of three independent experiments. $n=8$ for each group. $*P<0.05$. (right panel) Differential cell counts of BALF cells from asthmatic mice. Cells were spread on a glass slide, stained with Wright's solution, and differentially counted under light microscope. In both experimental groups, eosinophils comprised about two thirds of total cells, and the other one third was almost macrophages. Lymphocytes and neutrophils were observed with a very low frequency. These two groups showed no difference in cellular composition of BALF ($P>0.05$). Over 1,000 cells were counted in each group.

and the numbers of each immune cell including neutrophils, eosinophils, lymphocytes, and macrophage were differentially counted under light microscope. Majority of the cells from normal mice were macrophages (Fig. 3, left panels). In experimental groups, BALF cells were mainly composed of macrophages and eosinophils (middle and right panels in Fig. 3), which comprised 33.6% and 63.1%, and 20.2% and 65.4% of total cells in PBS-treated and vitamin C-treated groups respectively (Fig. 2, right panel). Occasional lymphocytes and neutrophils were also observed with a low frequency (2.9% and 0.4% in PBS-treated group, 3.7% and 0.7% in vitamin C-treated group). This compositional difference of cells between these two groups was statistically insignificant ($P>0.05$). Thus, vitamin C reduced total cell numbers in BALF, however, it did not affect the ratio of immune cells in BALF. Another finding that was observed was that macrophages from asthmatic mice revealed an activated appearance with a euchromatic nucleus, enlarged cell size, and cytoplasmic phagocytic vacuoles, while those from normal control mice showed quiescent appearance (lower panels in Fig. 3).

Vitamin C lowered the intensity of inflammatory cell infiltration around vessels and airways

One of the histological hallmarks of lung inflammation accompanying asthma is cellular infiltration around blood vessels and airway tracts (Duan *et al.*, 2004). To

evaluate whether vitamin C treatment during asthma induction affected perivascular and/or peribronchiolar cell infiltration, histological examination was performed. Lung tissues from normal mice showed no perivascular nor peribronchiolar inflammatory cell infiltration at all (Figs. 4A and D). Meanwhile, asthma induction resulted in cell infiltration around blood vessels and airway tracts. The cell infiltration was more prominent around blood vessels than around airways. The thickness of cell infiltration was semi-quantitatively evaluated by counting cell layers around the vessels or airways. As results, PBS-injected group showed perivascular infiltration of 6.4 ± 1.5 cell layer-thick in average, which was thicker than that for vitamin C-treated group (5.0 ± 2.9). This difference was statistically significant ($P<0.05$). The intensity of peribronchiolar cell infiltration also showed a tendency of reduction in vitamin C-treated group compared to PBS-injected group with average values of 1.2 ± 0.9 and 2.6 ± 1.8 , respectively ($P=0.058$).

One of the characteristic changes following asthma induction is mucus hyperplasia of respiratory epithelium (Izuhara *et al.*, 2009). Thus, we evaluated whether vitamin C treatment affected mucus hyperplasia. Lung tissues were PAS-stained and the degree of mucus hyperplasia was evaluated as the ratio of mucus-containing PAS-positive goblet cells per total respiratory epithelium (Figs. 4G~I). In normal mice, PAS-positive cells were hardly observed in the airway

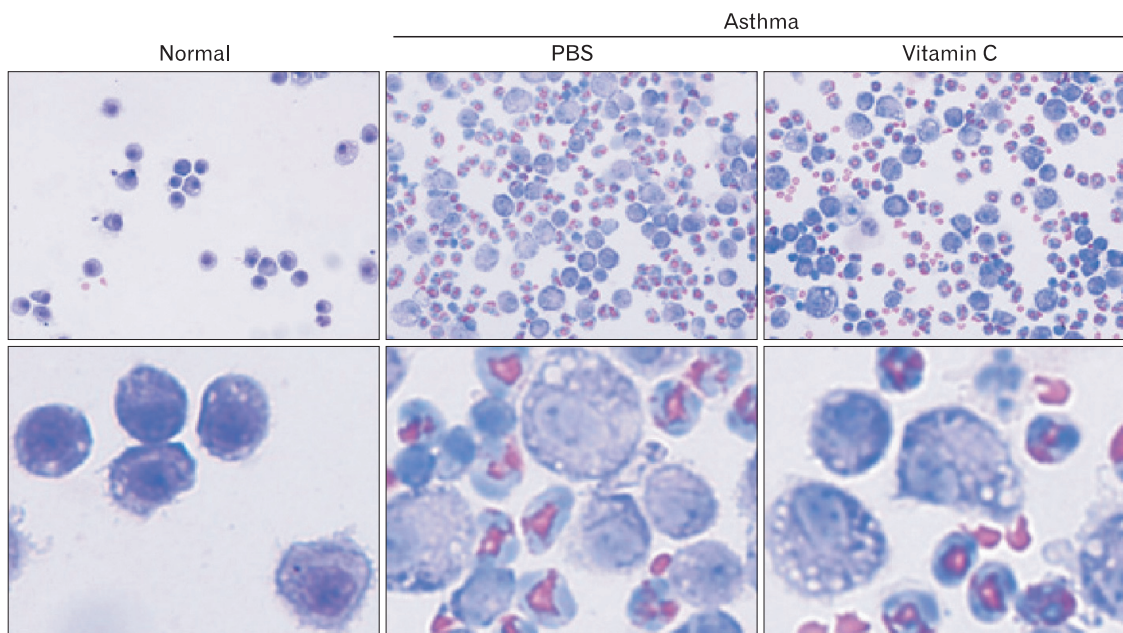


Fig. 3. Wright staining of BALF cells. After centrifugation of BALF, sedimented cells were re-suspended in 300 μ l PBS containing 0.05% albumin and monolayer cell spread was prepared by cytopsin. In normal mice, cells were scanty and almost all cells were macrophages with a quiescent appearance (left panels). Cells from mice with asthma, irrespective of PBS (middle panels) or vitamin C treatment (right panels), were abundant in number and mostly composed of eosinophils and macrophages. Macrophages in these groups differed from those of normal mice in their appearance and size. $\times 100$ magnification (upper panels) and $\times 800$ magnification (lower panels).

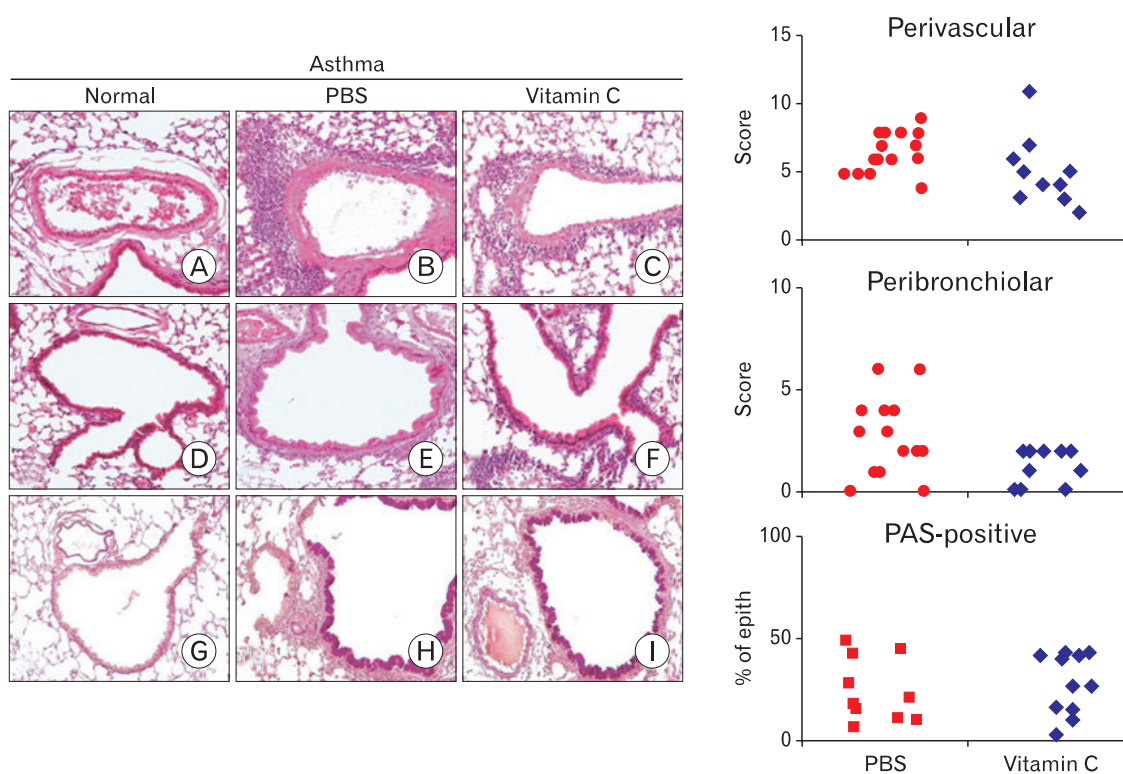


Fig. 4. Histological scoring of lung tissue. Paraffin sections were stained with hematoxylin and eosin (A~F) or with periodic acid-Schiff's reagent (G~I). The intensity of perivascular inflammatory cell infiltration (upper panels) or of peribronchiolar infiltration (middle panels) were semi-quantified as the numbers of cell layers and were depicted in graphs on the right column. Mucus hyperplasia were quantified as the ratio that goblet cells occupied on the respiratory epithelium and also depicted in the graph on the right column. Vitamin C administration significantly decreased the perivascular cell infiltration and tended to decrease peribronchiolar cell infiltration. However, the intensity of mucus hyperplasia was not affected by vitamin C treatment.

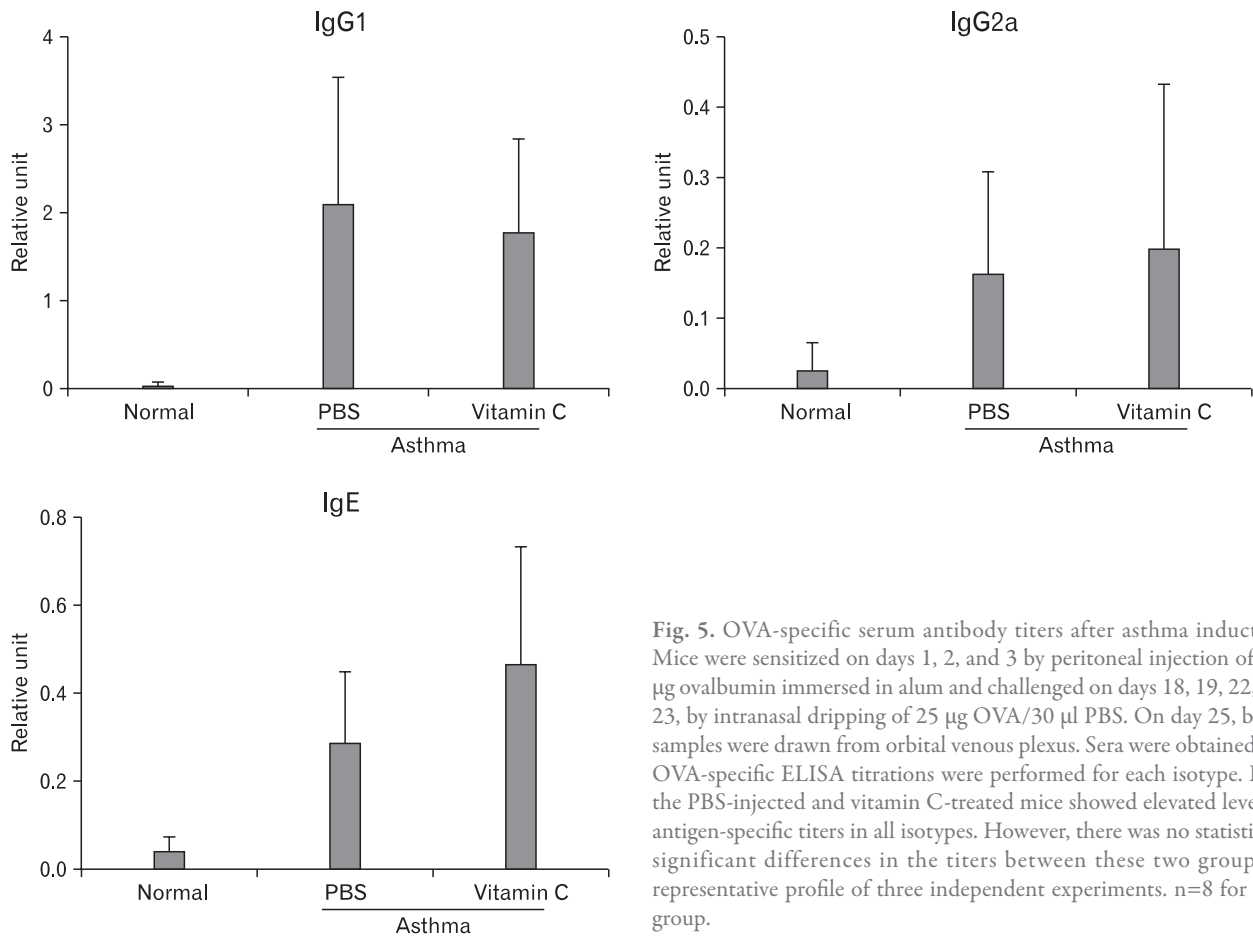


Fig. 5. OVA-specific serum antibody titers after asthma induction. Mice were sensitized on days 1, 2, and 3 by peritoneal injection of 100 μ g ovalbumin immersed in alum and challenged on days 18, 19, 22, and 23, by intranasal dripping of 25 μ g OVA/30 μ l PBS. On day 25, blood samples were drawn from orbital venous plexus. Sera were obtained and OVA-specific ELISA titrations were performed for each isotype. Both the PBS-injected and vitamin C-treated mice showed elevated levels of antigen-specific titers in all isotypes. However, there was no statistically significant differences in the titers between these two groups. A representative profile of three independent experiments. n=8 for each group.

epithelium from trachea to terminal bronchiole (Fig. 4G). In experimental animals, epithelia of airways, especially those of large ones, were covered by PAS-positive mucus secreting cells. In PBS-injected mice, goblet cells covered $25.7 \pm 15.3\%$ of all the respiratory epithelia in average, which was at a similar level to that for vitamin C-treated group ($28.5 \pm 14.8\%$). Thus, it looks like that vitamin C given at the time of antigen challenge did not affect mucus hyperplasia.

Vitamin C did not shift immune responses with regard to Th1/Th2

Ovalbumin-specific serum antibody titers for each isotype were measured (Fig. 5) to determine whether vitamin C shifted immune responses toward Th1 in asthma animal model. Results showed that OVA-specific antibody titers were not different in the two experimental groups in both Th1-dependent isotypes (IgG1 and IgE) and a Th2-dependent isotype (IgG2a). In addition, when the secretion of Th1 cytokines such as IL-12, IFN- γ , and TNF- α and Th2 cytokines such as IL-4 and IL-5 in the BAL fluid were measured, they

did not show any differences in both groups (data not shown).

Discussion

The aim of this study was to determine whether vitamin C administration did a beneficial role in animal asthma model. As results, vitamin C administered at a high dose at the time of antigen challenge reduced airway resistance provoked by methacholine. In addition, vitamin C reduced not only the number of inflammatory cells in BALF, but perivascular and peribronchial inflammatory cell infiltration. All these results together suggest that vitamin C attenuated lung inflammation induced by asthma.

Asthma has been regarded as a Th2-dependent disease, and Th2 cytokines such as IL-4, IL-5, and IL-13 with TGF β predispose and maintain this disease (Tagaya & Tamaoki, 2007; Broide 2008). Because a mega-dose vitamin C administered into the body shifts immune responses towards Th1 (Noh *et al.*, 2003; Noh *et al.*, 2005), we expected vitamin C administration at the time of antigen challenge in asthma

model would attenuate the disease by shifting immune response toward Th1. Actually, this idea has been mentioned by some authors. A high dose vitamin C administration was recently reported to decrease eosinophilic infiltration in ovalbumin-induced asthma mouse model (Chang *et al.*, 2009) and to decrease inflammatory cells in BALF, not only eosinophils but also neutrophils and lymphocytes in asthmatic guinea pig model (Haines *et al.*, 2010). In line with this concept, treatment using anti-cytokine agents or bacterial components aiming at the change of Th1/Th2 balance have been tried in moderate and severe asthmatics (Bjermer & Diamant 2004; Desai & Brightling, 2009). However, against the expectation, the results obtained in this experiment do not support Th1-shifting effects of vitamin C in asthma animal model. Vitamin C treatment did not shifted antigen-specific serum antibody isotype toward Th1 profile, neither changed cytokine profiles in BALF with respect to Th1/Th2 responses. In addition, cells in BALF from vitamin C-treated mice were composed mainly of macrophages and eosinophils just like in PBS-injected group. If Th1 shifting had been occurred by vitamin C administration, neutrophils were expected to prevail (Cohn *et al.*, 1998). Vitamin C given at the time of sensitization did not affected Th1/Th2 balance either. When we treated mice with vitamin C for 10 days during which mice were intraperitoneally sensitized with OVA, it exerted absolutely no effects on asthma including Penh, BALF cell number, and lung inflammation (unpublished data). These results are against what previously reported with delayed type hypersensitivity reaction induced by keyhole limpet hemocyanin (Noh *et al.*, 2005). In that experiment, administered mega-dose vitamin C clearly shifted immune responses toward Th1, nearly completely suppressing serum antigen-specific IgG1 and IgE antibody titers and rendering splenic T cells to secrete more IL-4 and less IFN- γ . We have no data to explain what made this discrepancy at this time. However, it has been reported that the immune response in animal asthma model varied depending on the route and frequency of antigen administration (Nelde *et al.*, 2001). Another possible clue to explain the discrepancy is that dendritic cells, which play an initial and principal role in Th1/Th2 shaping, have many subtypes which lodge differently from location to location in the body. These subtypes of dendritic cells respond differentially to a same antigen in different ways (Kirby *et al.*, 2001; Marleau *et al.*, 2008). That might direct immune responses against a same antigen differently according to the route through which antigens

enter the body. This point need to be further elucidated.

Even though no evidence of Th1 shifting was observed in this experiment, it is still true that the administered vitamin C attenuated the intensity of lung inflammation as evidenced by decreased cell numbers in BALF and less severe perivascular and peribronchial cell infiltration. These effects seems to be attributed to anti-inflammatory effects of vitamin C. Serum concentration of vitamin C has been supposed to be associated with inflammation in several epidemiological studies (Langlois *et al.*, 2001; Wannamethee *et al.*, 2006; Rizzo *et al.*, 2008). In addition, anti-inflammatory effect of vitamin C has been clinically applied to pancreatitis (Du *et al.*, 2003) with modest attenuation of serum inflammatory markers. These anti-inflammatory effects of vitamin C were ascribed to the anti-oxidant property of it. Or, some investigators insisted that vitamin C directly inhibited I κ B kinase phosphorylation leading to eventual inhibition of NF- κ B activation, which plays a critical role in inflammation (Cárcamo *et al.*, 2002).

However, this anti-inflammatory effect has been denied by some authors (Scott *et al.*, 2005; Hernandez *et al.*, 2009; Kamgar *et al.*, 2009). Thus, there are some controversies whether vitamin C is beneficial to inflammation. Our results support anti-inflammatory effects of vitamin C. That is, vitamin C given at the time of antigen challenge attenuated AHR and lung inflammation. However, the effect was not so much distinguished. Asthma is in nature a chronic inflammatory disease characterized by a phenominon known as "airway remodelling", which is contributed by subepithelial basement membrane thickening and fibrosis, increased smooth muscle cells, hyperplasia of mucus-secreting goblet cells, and so on (Hamid & Tulic, 2009). These changes takes time to occur and animal model of asthma usually does not reflect these changes satisfactorily because the disease model is not a chronic one (McMillan & Lloyd, 2004). Our model was also an acute one, and it should be necessary to evaluate the effects of vitamin C on the remodelling of airways in an established chronic type of asthma model (Zhou *et al.*, 2008).

In conclusion, even though vitamin C did not show any Th1/Th2 shifting effects in this experiment, it still exerted moderate anti-inflammatory effects. Furthermore, we can not still rule out that chronic usage of vitamin C would exert more beneficial effects on asthma. Considering other beneficial effects and that vitamin C is not an expensive one, we can regard mega-dose usage of vitamin C as a potential supplementary modality for the management of asthma.

Acknowledgements

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