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Article

Inhibitory Effect of Ore Minerals on the Allergic Inflammation in Mouse

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The ore minerals, mainly composed of sericite, talc, and halloysite, were investigated for their inhibitory effect on the allergic inflammation in mice by measuring the serum IgE level, inflammatory cytokine production, and histamine and β-hexosaminidase releases. When the mice were ovalbumin (OVA)-challenged prior to the ore mineral treatment, the IgE level in the serum decreased significantly. The ore minerals significantly reduced the productions of IL-4 and the tumor necrosis factor-α in the bronchial alveolar lavage fluid challenged with OVA. The inflammatory infiltrates, found in the lung of the OVA-challenged mouse, were also notably decreased following the ore mineral treatment. The ore minerals significantly reduced the release of histamine and β-hexosaminidase from the RBL-2H3 cells. These results suggest that the ore minerals may be useful as potent inhibitors of the allergic inflammation in mice.

Key words: *allergic inflammation, cytokine, histamine, IgE, ore minerals*

Over 20% of the human population suffers from allergic diseases such as atopic dermatitis, asthma, allergic rhinitis, and food allergy [Wuthrich, 1989]. Moreover, recently, the number of allergy sufferers has significantly increased due to the environmental pollution and the global warming [Yi *et al*., 2001]. Allergy is a pathological symptom due to the hypersensitive immune reaction. It is accompanied by inflammation and tissue damage, mediated by antibody or immune cells. The term 'allergy', which literally means 'altered reactivity' is often used interchangeably with 'hypersensitivity' [Jonathan *et al*., 2001]. Hypersensitivity responses can be classified into four types, based upon their underlying mechanisms and clinical manifestations [Averbeck *et al*., 2007]. Type 1 hypersensitivity reactions are immediate and involve allergen, allergen-specific IgE antibody, and mast cells. Type 2 hypersensitivity reactions, in contrast, are mediated by IgG or IgM antibody. Type 3 hypersensitivity reactions are also antibody-mediated, but the antigenic targets of Type 3 are soluble and not cell membrane-bound. Type 4 hypersensitivity reactions, in contrast to the Types 1-3, are cell-mediated reactions involving T lymphocytes and antigen-presenting cells.

Type 1 is the most typical allergy type, an immediate hypersensitive reaction involving mast cell, allergen, and allergen-specific IgE antibody. The clinical manifestations of Type 1 hypersensitivity include, among others, allergic rhinitis, bronchial asthma, eczema, and hay fever. The most severe form of Type I reaction is the systemic anaphylaxis, a life-threatening condition. Upon exposure to allergens, IgE antibody on the surface of the mast cells bind allergens via their antigen-binding (Fab) portions and become cross-linked with the Type I response. The mast cell activation increases the calcium concentration within the cell to provoke degranulation, thereby causing the release of the allergy mediators [White *et al*., 1985]. Allergy mediators isolated from the mast cells include not only histamine, protease, leukotriene, and prostaglandin, but also numerous proinflammatory cytokines and chemotaxis cytokines such as TNF-α, IL-4, IL-6, IL-13, and TGF-β [Metcalfe *et al*., 1981; Burd *et al*., 1989; Plaut *et al*., 1989; Bradding *et al*., 1993]. These various

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Abbreviations: BAL, bronchial alveolar lavage; BSA, bovine serum albumin; DMEM, Dulbecco's modified eagle's medium; DNP, dinitrophenol; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; H&E, haematoxylin and eosin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; i.p., intraperitoneally; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPT, *o*-phthalaldehyde; OVA, ovalbumin; PBS, phosphate buffered saline; Th1, Type 1 T helper; Th2, Type 2 T helper; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

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mediators interact with the surrounding cells to provoke allergic reaction, and, among the mediators, histamine is a powerful vasodilator, which mostly affects the Type 1 allergy [Petersen *et al*., 1996]. Also, β-hexosaminidase, which exists in the secretory granule of the mast cells that store histamine, is released along with histamine when the mast cell is immunologically activated [Xu *et al*., 2006]. Both histamine and β-hexosaminidase are degranulation indicators that determine the extent of degranulation degree and are widely used for the assessment of anti-allergy effect [Hoffmann *et al*., 1999].

It is generally known that the Th1 cells produce IFN-γ, IL-2, IL-6, IL-10, IL-12, IL-18, IFN-α, and IFN-β as well as TNF-beta, which lead to the cell-mediated responses such as macrophage activations and are responsible for the cell-mediated immunity and the phagocyte-dependent protective responses. By contrast, Th2 cells produce IL-4, IL-5, IL-9, and IL-13, which are responsible for such humoral immune responses as the antibody production, the eosinophil activation, and the inhibition of several macrophage functions, thus providing the phagocyte-independent protective responses. Accordingly, allergy results from an imbalance in favor of a Th2 response and is negatively regulated by Th1 cells. Therefore, Th1/Th2 paradigm has provided the rationale for the therapy of allergy; hence, the regulation of Th1/Th2 cells is necessary to treat allergies.

There are several reports on natural products that effectively suppressed compound 48/80-induced histamine release and passive anaphylaxis reaction [Yi *et al*., 2001; Kim *et al*., 2006]. In the present study, the ore minerals, mainly composed of medicinal minerals that are known to be effective for treating diseases according to *Dongeuibogam* (Encyclopedia of Eastern Medicine), the most celebrated Korean materia medica published about 400 years ago, were used to investigate their inhibitory effects on the allergic inflammation. The effects of ore minerals on the allergic inflammation in the OVA-challenged mice were investigated focusing on the serum IgE concentration, the cytokine concentration in BAL, and the histological characteristics of the lung.

Materials and Methods

Animal and ore mineral preparations. BALB/c male mice were obtained from the Orient Bio Co. Ltd (Seoul, Korea). Mice, 7 weeks old, each weighing 18-19 g were acclimatized for 2 days at 25±2°C and the normal day/night cycle before starting the experiment. The ore minerals used to investigate the efficacy of the allergic inflammation were developed by NT&BT Co. Ltd (Hongsung, Korea). The manufacturing processes of the minerals were described in our previous report [Kang and Lee, 2008].

Sensitization and challenge. The mice were sensitized with 0.2 mL of a normal saline containing 500 μg/mL OVA (Sigma, St. Louis, MO) adsorbed on 100 mg/mL aluminum hydroxide i.p. on days 0 and 14. Seven days after the final i.p. injection, the mice were administered the mineral sample orally on days 21, 22, and 23. After 30 min of the oral administration, 100 μL OVA (150 μg/100 μL) inhalation was performed [Park *et al*., 2007].

Measurement of OVA-specific IgE. On day 24, all mice were sacrificed, and the blood samples were collected. The sera were separated by centrifugation at 13,000 rpm for 10 min and kept at −70°C until IgE analysis. The OVA-specific IgE levels in the sera were measured by ELISA. The results were expressed in ng/mL of serum. The plates were coated with the diluted anti-mouse IgE overnight at 4°C, followed by the addition of 1:250 diluted sera diluted with the phosphate-buffered saline containing 0.05% Tween-20 into the wells, and the plates were incubated for 2 h at RT. The bound IgE was detected with the biotinylated anti-mouse IgE antibodies. The plates were then developed by the addition of horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine and read in the ELISA plate reader at 450 nm.

Cytokine determination. Shortly after exsanguination, the trachea was cannulated, and 1 mL saline was used per lavage and repeated four times for each mouse. About 4 mL of the BAL fluid was centrifuged at 12,000 rpm for 10 min, and the supernatant was kept at −70°C until analysis for cytokines. IL-4 and TNF-α were measured by a modified ELISA. The ELISA was performed in the 96-well plates coated with the capture antibody at 4°C overnight, and the coated plates were washed with the phosphate-buffered saline containing 0.05% Tween-20. Assay plates were exposed to the biotinylated 2nd antibody, the horseradish peroxidase, and the 3,3',5,5'-tetramethylbenzidine substrate solution. The plates were read at 450 nm in an ELISA reader, and the cytokine levels were measured for comparison with the standard.

Lung histopathology. The lungs were gently filled with 1 mL of 4% neutral-buffered paraformaldehyde and then immersed in the paraformaldehyde solution for fixation. The lungs were subsequently embedded in paraffin, sectioned at a thickness of 4 μm, and stained with H&E for light microscopy [Oh *et al*., 2007]. Eosinophiles were determined by the point-counting technique at 400× magnification across 10 random non-coincident microscopic fields, and the averages of the numeral eosinophiles were calculated.

MTT assay for cell viability. The MTT assay was used to determine the maximum concentration of the

extract at which the cell viability was not affected. In brief, the cells were cultured in a 96-well plate (Corning Inc, Corning, NY) at a density of 3×10^6 cells/mL. The cells were then treated with varying concentrations of the ore minerals. After 24 h, the cells were washed and treated with MTT. The plates were incubated in the dark for 2 h at 37°C. After the formazan formation, 100 μL/well dimethyl sulfoxide was added, and the absorbance was measured at 570 nm using a microtiter plate reader (Molecular Devices Co., Sunnyvale, CA) [Jeon *et al*., 2007; Lee *et al*., 2008].

Histamine assay. Rat basophile leukemia cell line RBL-2H3 was maintained in the DMEM medium with 10% FBS and 100 unit/mL penicillin/streptomycin at 37°C under 5% CO_2 in the air. The cells (2×10^4 cells/well) were precultured at 37°C for 24 h in a 96-well plate containing 0.1 mL/well of the medium. The supernatants were discarded, and the cells were incubated at 37°C for 2 h with DMEM containing 2% FBS and anti-DNP IgE. The cells were washed three times with the HEPES buffer. After incubation in 0.1 mL of the HEPES buffer containing the ore minerals at 37°C for 10 min, the cells were challenged with DNP-BSA (4 μg/200 μL) at 37°C for 35 min. The plate temperature was lowered to 4°C to stop the reaction. Five minutes after the supernatant was moved to a 24-well plate, 0.2 mL of 1 N NaOH and 0.1 mL of 1% OPT were added at room temperature. The reaction was stopped with the addition of 0.2 mL/well of 0.1 N HCl. The plate was respectively read at 405 and 450 nm of excitation and emission in a fluorometer. The analyses were performed in triplicate, and the results were expressed as the percentage of the total release minus the spontaneous release.

β-Hexosaminidase assay. β-Hexosaminidase assays were performed under the same cell culture condition as used in the histamine assay. After stimulation by DNP-BSA, the cells were spun at 5,000×g for 1 min, and the supernatants were collected and chilled on ice. Fifty microliters of each sample was incubated with 50 μL of 1 mM *p*-nitrophenyl-β-acetyl-D-glucosamide (Sigma, St. Louise, MO) dissolved in 0.1 M citrate buffer (pH 5) in a 96-well microtiter plate at 37°C for 1 h. The reaction was stopped by the addition of 200 μL/well of 0.2 M glycine buffer (pH 10.7). The plate was read at 407 nm in an ELISA reader.

Statistical analysis. Values are expressed as the means ±SEM. Differences between the groups, OVA-, OVA+/mineral- and OVA+/mineral+ were determined by analysis of variance, followed by Duncan's multiple-range test for all pairwise multiple comparisons. A p value <0.05 was considered to be statistically significant.

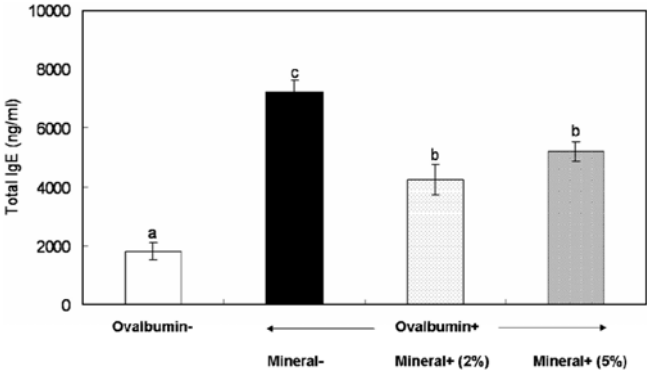

Fig. 1. Effect of ore mineral treatment on the IgE level in the mouse serum. a, c: $p<0.05$ versus the OVA sensitized mice.

Results and Discussion

Reduction of serum IgE concentration. It is well known that the notable increase in the IgE production is facilitated to provoke allergic inflammations, namely asthma, atopy, and eczema. This unusual IgE production in the serum is caused by the activation of Th2 cell, which, in turn, results in the secretion of IL-4, IL-5, IL-9, and IL-13 [Lee *et al*., 2003; Fish *et al*., 2005; Kim and Byun, 2005], among which IL-4 mostly regulates the IgE production.

The effects of the ore minerals on the serum IgE concentration of the OVA-challenged mice are shown in Fig. 1. The OVA is reported to trigger a persistent allergic inflammation in mice [Jeong *et al*., 2005]. The IgE concentration of the OVA-challenged control was 7250.75 ng/mL, four times higher than that of the normal PBS-treated IgE. However, this increase in the OVA-challenged control was significantly lowered to 4255.77 ng/mL by the 2% ore mineral treatment. This result showed that the ore mineral mixture decreased the serum IgE concentration of the OVA-challenged mice by 40%, indicating that the ore mineral mixture is effective in suppressing the OVA-induced serum IgE concentration; the result of the 5% ore mineral treatment was not significantly different from that of the 2% treatment.

Reduction of cytokoline in bronchoalveolar lavage fluid. Cell components of the lower respiratory tract can be obtained from BAL of the mice with the respiratory diseases. Especially during the asthma attack, the expression of the Th2 cytokines in the BAL fluid increases. Among the Th2 cytokines in BAL, IL-4 plays an important role in causing the allergic inflammation via enhancement of the IgE production. IL-4 is also known as a major cytokine that causes chronic inflammation [Sy *et al*., 2006]. Furthermore, IL-4 induces vascular cell adhesion molecule-1

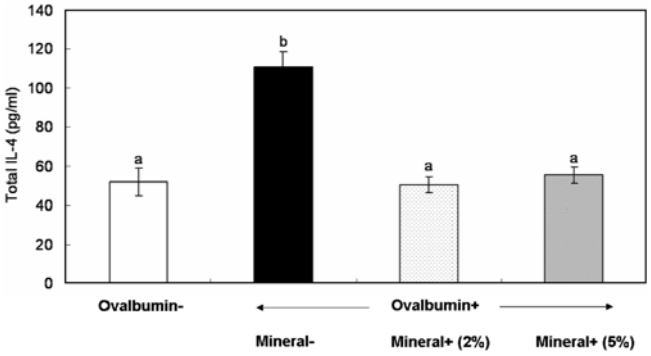

Fig. 2. Effect of ore mineral treatment on the interleukin-4 (IL-4) level in bronchoalveolar lavage fluid. a: $p<0.05$ versus the OVA sensitized mice.

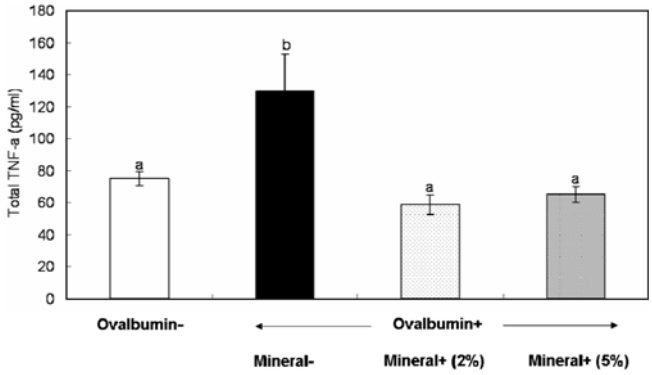

Fig. 3. Effect of ore mineral treatment on the TNF-α level in bronchoalveolar lavage fluid. a: $p<0.05$ versus the OVA sensitized mice.

gene expression in the endothelium and gathers the eosinophils to the inflammatory lesions to cause increased infiltration, leading to a chronic inflammation.

TNF-α, a pivotal proinflammatory cytokines increased by the allergic inflammation, is released from both the mast cells and the macrophages via IgE-dependent mechanisms in the allergic responses, and the elevated levels of TNF-α are frequently observed in the bronchoalveolar fluid of the asthmatic subjects undergoing allergen challenge [Kim *et al*., 2006]. The TNF-α expression mediates the neutrophil migration and infiltration. Furthermore, it not only increases the particle-induced cytotoxicity, but also regulates the neutrophil apoptosis in the acute inflammation. Th2 cytokines such as IL-4, IL-13, as well as TNF-α, are continuously released during the airway remodeling in the allergic inflammation.

Figure 2 shows the IL-4 concentration changes in the BAL of the OVA-challenged mice. IL-4 concentration of the OVA-challenged control was 110.7 pg/mL, two-fold higher than that of the PBS treatment. Increased IL-4 level of the control was significantly lowered to the PBS treatment level after the ore mineral treatment.

TNF-α concentration of OVA-challenged control was 129.52 pg/mL, a 1.7-fold increase compared to 75.01 pg/mL of the PBS treatment. Increased TNF-α concentration of the control decreased about 50% compared with the other controls after 2 and 5% ore mineral treatments (Fig. 3). These results showed that the ore mineral mixture effectively lowered the concentrations of OVA-challenged IL-4 and TNF-α in BAL.

Effect of ore minerals on the lung inflammation. The H&E-stained lung tissues were evaluated for inflammatory lesions. In general, the eosinophil number is determined to diagnose the allergic inflammation, because a marked infiltration of the inflammatory eosinophil is observed in the allergic inflammation. The lung sections belonging to the OVA-challenged group showed a significant increase of the pulmonary inflammation, with 37 eosinophils observed (Fig. 4). Interestingly, no inflammation was observed in the ore mineral-treated group. The inflammatory eosinophil number in the ore mineral group decreased to one, similar to that of the normal group (Table 1). The number of eosinophils is the average of 10 views per sample as observed by a 400× magnification optical microscope. These results suggest that ore minerals strongly reduce the inflammatory eosinophils in the OVA-challenged lung tissue of the mice.

Effects of ore mineral treatment on secretion of histamine and β-hexosaminidase. RBL-2H3, a rat basophilic leukemia cell line, is a mucosa mast cell [Nakatani *et al*., 2002] that is widely used in the IgE-mediated degranulation research [Barsumian *et al*., 1981; Metzger, 1992]. IgE antibody receptor exists on the surface of the RBL-2H3 cell, and histamine and β-hexosaminidase located inside RBL-2H3 are released by the cross-linking of the antigen-induced IgE receptor. This leads to the mast cell activation by enhancing the calcium ion influx, and the release of inflammation mediators such as TNF-α and IL-1 to cause allergic inflammation [Benhamou *et al*., 1993; Yamashita *et al*., 2000].

When the effects of the ore minerals on the survival and the proliferation of RBL-2H3 were observed, the highest concentration of the ore minerals at which the cell survival and the proliferation was not affected was 0.25% (data not shown). The ore mineral treatments of RBL-2H3 at 0.25 and 0.125% reduced the histamine secretion by about 80 and 50%, respectively (Fig. 5). These results indicate that the increasing of the ore mineral concentration significantly lowered the histamine secretion in the RBL-2H3 cell. Figure 6 shows the effect of ore minerals on the

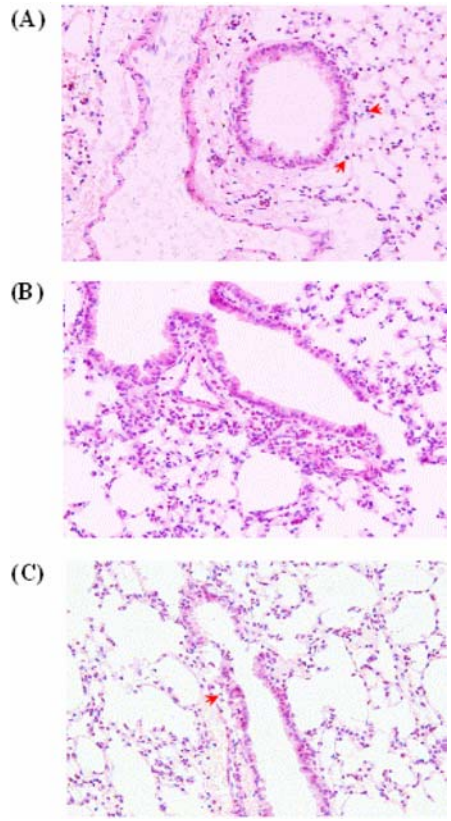

Fig. 4. Effect of ore minerals on the histological changes of lung tissue from BALB/c mouse. Representative photomicrographs of lung sections from normal mice (A), OVA-treated mice (B), mineral-treated mice (C). Arrow indicates infiltrated eosinophils in the lung tissue.

Table 1. Numbers of infiltrated eosinophils in lung tissue of BALB/c mouse

	No of eosinophil*
Ovalbumin-	2
Ovalbumin+	37
Ovalbumin+/Mineral+	1

*high power field (×400)

anti-DNP IgE-mediated β-hexosaminidase release in the RBL-2H3 cell. The β-hexosaminidase secretion was respectively lowered by 30 and 20% compared to that of the control after 0.25 and 0.125% ore mineral treatments.

Taken together, the ore mineral treatment could reduce the serum IgE level as well as those of IL-4 and TNF-α of the BAL fluid in the OVA-challenged mice. Moreover, the eosinophil count was reduced to the normal level after the ore mineral treatment. Furthermore, histamine and β-hexosaminidase secretions in RBL-2H3 were reduced after the ore mineral treatment, indicating that ore minerals effectively inhibit the allergic inflammation of the OVA-challenged mice.

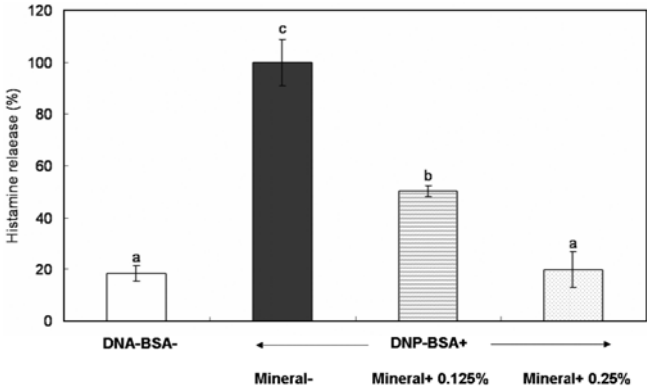

Fig. 5. Effect of ore minerals on the anti-DNP IgE-induced histamine release from RBL-2H3 cell. a, b: $p<0.05$ versus the DNP-BSA stimulation group.

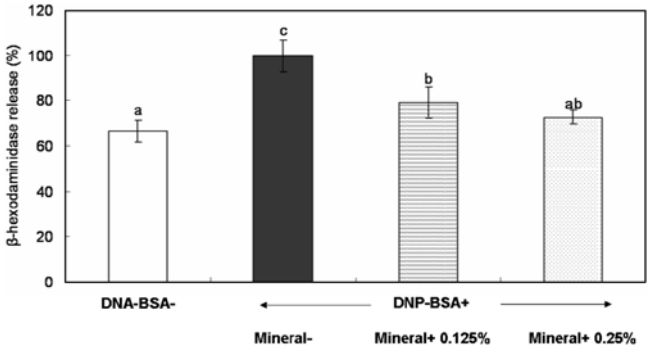

Fig. 6. Effect of ore minerals on the anti-DNP IgE-induced β-hexosaminidase release from RBL-2H3 cell. a, b: $p<0.05$ versus the DNP-BSA stimulation group.

Among the 92 types of ore minerals listed in *Dongeuibogam*, sericite, halloysite, muscovite, and talc are well known to contain healthy inorganic materials such as selenium, iron, and calcium. However, although the pharmacological effects of these medicinal ore minerals are partly known, literatures available to substantiate these effects scientifically are scarce. The ore mineral mixture used in the present study, composed mainly of sericite and halloysite, was processed into powder using different methods. The mineral mixture showed an anti-wrinkle improvement effect through the reduction of matrix metalloproteinases [Kang and Lee, 2008] in the UV-irradiated human dermal fibroblast cell, and a wound-healing effect in mice [Choi *et al*., 2008], possibly due to the antioxidant activity [Kang and Lee, 2008] of the ore minerals. In the present study, the inhibitory effect of the ore mineral treatment on the allergic inflammation in mice was evaluated. The results suggest that, after toxicity and safety assessments, the ore minerals may be utilized as an effective material for the improvement of the allergic inflammation in diverse fields.

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