Regular Article*Highlighted Paper selected by Editor-in-Chief***Effects of Supplementary Seleno-L-methionine on Atopic Dermatitis-Like Skin Lesions in Mice**Tomohiro Arakawa,*^a Takahiro Sugiyama,^a Haruka Matsuura,^a Tomofumi Okuno,^a Hirofumi Ogino,^a Fumitoshi Sakazaki,^b and Hitoshi Ueno^a^aDepartment of Public Health & Preventive Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University; 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan; and ^bLaboratory of Toxicology, Faculty of Pharmacy, Osaka Ohtani University; 3-11-1 Nishikiori-Kita, Tondabayashi, Osaka 584-8540, Japan.

Received May 9, 2018; accepted June 25, 2018

Effects of selenium supplementation on atopic dermatitis (AD) were investigated by administering seleno-L-methionine (SeMet) using a mouse model of AD caused by repeated application of 2,4,6-trinitrochlorobenzene (TNCB). BALB/c mice were sensitized with TNCB to the abdomen on day -7; then, TNCB was applied repeatedly to each ear three times a week from days 0 to 23. SeMet was orally administered to the mice from days 0 to 23. The efficacy of SeMet on AD was assessed by measuring ear thickness, histologic evaluation, serum total immunoglobulin (Ig) E levels, and expression of interleukin (IL)-4 in the ear and superficial parotid lymph node. Ear thickness was remarkably increased by repeated application of TNCB, and SeMet significantly suppressed ear thickness in BALB/c mice. SeMet inhibited epidermal hyperplasia and dense infiltration of inflammatory cells. The number of TNCB-induced mast cells was significantly decreased by SeMet. Serum total IgE levels that increased by the repeated application of TNCB were significantly suppressed by SeMet. Repeated application of TNCB induced expression of IL-4, a T-helper (Th) 2 cytokine, in the ear and superficial parotid lymph node of BALB/c mice and its expression was significantly inhibited by SeMet. These results demonstrated that SeMet supplementation suppresses AD-like skin lesions in BALB/c mice and inhibits the expression of total IgE and IL-4.

Key words atopic dermatitis; seleno-L-methionine; immunoglobulin E; interleukin 4; 2,4,6-trinitrochlorobenzene

The prevalence of atopic dermatitis (AD), a chronic inflammatory skin disease, has substantially increased over the past 30 years; currently, 15–30% of children and 2–10% of adults suffer from AD, leading to a significant reduction in the QOL and increase in economic burden.^{1,2)} The disease is characterized by intense itching and eczematous lesions, and immunoglobulin (Ig) E-mediated reactions are known to cause inflammation.³⁾ Several reports have demonstrated that type 2 cytokine levels, including interleukin (IL)-4 levels, are increased in patients with AD.^{4–6)} IL-4 produced by T cells has a central role in the recruitment of inflammatory cells in AD.

Selenium, an essential trace element for the health of humans and animals, is attained through consumption of various dietary components.⁷⁾ Selenium is incorporated into selenoproteins, and the biological effects of selenium are exerted primarily through the function of different selenoproteins. Glutathione peroxidase (GPx) and thioredoxin reductase (TR), which are well-known selenoproteins, are important in the cellular antioxidant defense system.⁸⁾ Primary dietary selenium sources include wheat and yeast, which contain seleno-L-methionine (SeMet).^{9,10)} SeMet is one of the organic selenium compounds and is contained in various foods and is reportedly one of the main chemical forms of dietary selenium.^{7,11)} SeMet is considered a useful chemical form for nutritional supplementation compared with inorganic selenium compounds.^{12–15)}

Some studies have reported that patients with asthma showed significantly lower selenium concentration in nails or blood and significantly lower GPx activity in the blood.^{16–20)} According to a cohort study on AD prevention, selenium deficiency has been reported to increase the risk of AD.²¹⁾

Moreover, increased oxidative stress has been reported to be involved in the pathogenesis of AD.^{22,23)} Based on these reports, it is speculated that the supplementation of selenium compounds might be effective for preventing AD. However, the effects of selenium supplementation on AD remain unknown.

Repeated applications of 2,4,6-trinitrochlorobenzene (TNCB) to mouse skin have been reported to induce AD-like skin lesions, which are associated with a significant increase in serum IgE and T-helper (Th) 2 cytokines, such as IL-4, at the chronic dermatitis site.^{24–27)} In this TNCB-induced AD mouse model, the inflammatory response in chronic lesions has been reported to share many of the histopathological, immunological, and clinical features of human AD.^{24,28)}

We clarified and assessed the effects of SeMet supplementation on AD in a mouse model of chronic dermatitis caused by repeated TNCB application.

MATERIALS AND METHODS

Animals Five-week-old female BALB/c mice were purchased from Japan SLC Co. (Shizuoka, Japan) and rested for 1 week after arrival. The mice were treated and kept in a specific pathogen-free room maintained at 23±1°C and 47–67% humidity, under a 12h light–dark cycle, and given a normal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and distilled water *ad libitum*. The experimental protocol used was in accordance with the animal experimental guidelines of Setsunan University as modified from the guidelines of the Japanese Society for Pharmacology. This experiment was approved by

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Fig. 1. Experimental Schedule for Induction of AD-Like Skin Lesions

TNCB was applied to abdominal skin of mice on day -7, and then repeatedly to each ear three times a week on days 0 to 23. SeMet was administered orally on days 0 to 23.

the Committee for the Ethical Use of Experimental Animals of Setsunan University. Throughout the experimental procedures, every effort was made to minimize animal suffering as much as possible by limiting the number of animals used in this study.

Reagents SeMet was purchased from Acros Organics (Geel, Belgium). TNCB (purchased from Tokyo Kasei Co., Tokyo, Japan) was dissolved in acetone-olive oil (3:1) as a 0.1% (w/v) solution, and used for sensitization of mice and challenge of AD-like skin lesions.

Experimental Procedure Mice were sensitized by a single epicutaneous application of 50 μ L TNCB solution to the shaved abdomen on day -7. Then, 10 μ L/ear TNCB solution was applied repeatedly to each ear three times a week through days 0 to 23. As a negative control, vehicle (acetone-olive oil [3:1]) was applied to both ears instead of TNCB. SeMet (TNCB+SeMet group) or saline (vehicle and TNCB-only group) was administered orally once daily on days 0 to 23. Ear thickness was measured using a digital thickness gage (Ozaki MFG. Co., Tokyo, Japan) at 0, 3, 6, and 24 h after each challenge. The serum was collected from the mice on days 0, 6, 13, 20, and 24. On day 24, 24 h after the final TNCB challenge, the mice were sacrificed and their ears, superficial parotid lymph nodes, and liver were collected. The design of the experimental schedule is summarized in Fig. 1.

Measurement of Selenium Concentration Liver tissues were digested at 80°C for 1 h, then at 140°C for 2 h, and finally at 170°C for 30 min in 1 mL of a 1:2 mixture of nitric acid and perchloric acid. Selenium concentration was measured by the fluorometric method using 2,3-diaminonaphthalene.²⁹⁾

Histological Evaluation A piece of the skin was removed, fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), embedded in paraffin, and cut into 5- μ m thick sections. One of the sections was stained with hematoxylin (Mayer's Hematoxylin Solution; Merck, Darmstadt, Germany) and eosin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), whereas the other one was stained with toluidine blue (Wako Pure Chemical Industries, Ltd.) for the detection of mast cells. The number of mast cells was expressed as previously described.³⁰⁾ Briefly, mast cells were counted using microscopic observations on 6 to 8 consecutive fixed fields of view along the entire length of the ear skin and were calculated per mm cartilage length.

Measurement of Serum IgE Level Serum was collected from the mice on days 0, 6, 13, 20 and 24, and stored at -80°C until just before use. Serum IgE levels were determined using a commercially available mouse IgE enzyme-linked immunosorbent assay (ELISA) Kit (BioLegend, CA,

U.S.A.). Assays were performed according to the manufacturer's instructions.

RNA Extraction and Real-Time Quantitative PCR Total RNA was extracted from ear and superficial parotid lymph node using the Sepasol Reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. An aliquot of total RNA (1 μ g) in each sample was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, U.S.A.). Obtained cDNA was used for real-time quantitative PCR with the LightCycler 480 System II (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR Green I Master Mix (Roche Diagnostics GmbH). Samples were predenatured at 95°C for 5 min, denatured at 95°C for 10 s, annealed at 60°C for 10 s, and elongated at 72°C for 10 s for 45 cycles, followed by melting curve analysis between 65 and 95°C to confirm the absence of non-specific amplification. Results were analyzed using Relative Quantification Software (Roche Diagnostics GmbH). Primer sequences used were: IL-4 forward: 5'-TCTCGAATGTAC CAGGAGCCATATC-3'; IL-4 reverse: 5'-AGCACCTTG GAAGCCCTACAGA-3'; ribosomal protein S18 (Rps18) forward: 5'-TTCTGGCCAACGGTC TAGACAAC-3'; and RPS18 reverse: 5'-CCA GTGGTCTTG GTGTGCTGA-3'. Results were expressed as the mRNA level relative to Rps18 mRNA as an internal control.

Measurement of IL-4 Protein IL-4 was measured as previously described,³¹⁻³³⁾ but with a slight modification. Briefly, punched ear (8 mm in diameter) and one superficial parotid lymph node were collected 24 h after the final TNCB challenge. The specimens were homogenized in 0.5 mL of ice-cold phosphate buffered saline containing 0.1% Tween 20 by rapid agitation for 1 min in the presence of 2-mm zirconia beads using Micro Smash MS-100 (TOMY SEIKO Co., Ltd., Tokyo, Japan), and then centrifuged for 10 min at 12000 $\times g$ in a cold microfuge. Each supernatant was quantified using an ELISA kit for IL-4 (BioLegend). Assays were performed according to the manufacturer's instructions.

Statistical Analysis Statistical analysis was performed using one-way ANOVA with a Bonferroni *post hoc* test. In the figures, ** and * indicate statistical significance at *p* values of 0.01 and 0.05, respectively.

RESULTS

Effects of SeMet on Ear Thickness in an AD Model with Repeated Application of TNCB Mice were sensitized with TNCB on day -7, and then TNCB solution was applied repeatedly to each ear on days 0, 2, 5, 7, 9, 12, 14, 16, 19, 21,

and 23 (Fig. 1). SeMet was orally administered to mice on days 0 to 23 (Fig. 1). As shown in Table 1, selenium concentration in liver increased following SeMet supplementation in

Table 1. Selenium Concentration in the Liver

	Selenium concentration ($\mu\text{g/g}$)
Vehicle	1.59 ± 0.11
TNCB-only	1.62 ± 0.13
TNCB+SeMet ($5 \mu\text{mol/kg/d}$)	$2.34 \pm 0.63^*$
TNCB+SeMet ($10 \mu\text{mol/kg/d}$)	$3.88 \pm 0.33^{**}$

The results are presented as mean $\pm$ standard deviation (S.D.) ($n=6$). $^{**}p<0.01$, $^*p<0.05$, compared with the TNCB-only group.

a dose-dependent manner. Ear thickness was measured at 0, 3, 6, and 24h after each challenge (Fig. 2). Peak ear thickness appeared at 24h after TNCB application on days 0 to 5, and at 6h on days 7 to 23 (Fig. 2A). These results showed a shift in the time course of peak ear thickness from 24h after TNCB application to 6h by repeated application of TNCB. SeMet supplementation suppressed ear thickness in a dose-dependent manner. Ear thickness at 6h after the each TNCB application (early-type response) gradually increased with repeated application (Fig. 2B). Although ear thickness in mice administered $10 \mu\text{mol/kg/d}$ SeMet also gradually increased with repeated application, the degree of the increase was suppressed over the whole period. Similarly, ear thickness at 24h after each application (delayed-type response) also gradually increased

Fig. 2. Effects of SeMet on Ear Thickness after Repeated Application of TNCB

Ear thickness was measured at 0, 3, 6, and 24h after each TNCB challenge on days 0 to 23. (A) 0, 3, 6, and 24h after each TNCB challenge. (B) At 6h after each TNCB challenge. (C) At 24h after each TNCB challenge (○, vehicle; ●, TNCB; ▽ and ▼, TNCB+SeMet 5 and $10 \mu\text{mol/kg/d}$, respectively). The results are presented as mean $\pm$ S.D. ($n=12$). $^{**}p<0.01$, $^*p<0.05$, compared with the TNCB-only group. Experiments were repeated independently at least three times and similar results were obtained.

Fig. 3. Histologic Features of the Ears

Ear sections on day 24 were stained with hematoxylin and eosin to show cellular infiltration and tissue structure. Scale bar: 50 μ m. Representative data are shown. Experiments were repeated independently at least three times and similar results were obtained.

with repeated applications (Fig. 2C). Ear thickness in mice administered 10 μ mol/kg/d SeMet (TNCB+SeMet group) was less than that in mice administered TNCB only. On the contrary, repeated treatment with vehicle (negative control group) had no detectable effect.

Morphologic Ear Changes The inflammatory features in the ear were analyzed histologically (Fig. 3). Increased ear thickness, epidermal hyperplasia, and significant inflammatory cell infiltration were observed in TNCB-treated ears compared with vehicle. SeMet supplementation markedly reduced TNCB-induced increased ear thickness, epidermal hyperplasia, and significant inflammatory cell infiltration.

The number of mast cells in the ear (measured with toluidine blue staining; Fig. 4) was significantly increased in the TNCB-only compared with the negative control (vehicle) groups (Fig. 4B). The number of TNCB-induced mast cells was significantly decreased by SeMet supplementation (TNCB+SeMet groups).

Serum Total IgE Levels To evaluate the changes in total IgE level associated with progression of TNCB-induced AD, we collected serum on days 0, 6, 13, 20, and 24. Serum total IgE levels, measured by ELISA, increased with repeated TNCB application (Fig. 5). A relatively steep increment in total IgE levels was observed from days 6 to 13, almost reaching a plateau at day 13. In the TNCB+SeMet group, total IgE levels were lower at any point compared with the TNCB-only group, increased until day 13, and then decreased until day 24. TNCB-induced IgE elevation was suppressed significantly by SeMet in a dose-dependent manner.

IL-4 Expression in Ear and Superficial Parotid Lymph Node Expression of mRNA for IL-4 in ear and superficial parotid lymph node at 24h after the final TNCB challenge on day 24 was measured using real-time quantitative PCR. IL-4 mRNA levels in ear and superficial parotid lymph node were significantly increased by repeated TNCB application. TNCB-induced IL-4 mRNA expression in the ear was inhibited significantly in mice administered 10 μ mol/kg/d SeMet (Figs.

Fig. 4. Number of Mast Cells

Dermal mast cells (arrows) were stained with toluidine blue and the number of mast cells was counted. (A) A representative staining feature is shown. Scale bar: 100 μ m. (B) Number of mast cells per mm cartilage length. The results are presented as mean $\pm$ S.D. ($n=6$). ** $p<0.01$, compared with the TNCB-only group. Experiments were repeated independently at least three times and similar results were obtained.

6A, B). IL-4 mRNA expression in superficial parotid lymph node was inhibited significantly in mice administered 5 or 10 μ mol/kg/d SeMet.

We also measured the production of IL-4 in ear and superficial parotid lymph node at 24h after the final TNCB challenge on day 24 using ELISA (Figs. 7A, B). IL-4 protein production in ear and superficial parotid lymph node was increased significantly by repeated application of TNCB compared with that in the vehicle group. IL-4 production was inhibited significantly at both locations in mice given 10 μ mol/kg/d SeMet. These results corresponded with results of IL-4 mRNA expression in ear and superficial parotid lymph node.

DISCUSSION

Using a TNCB-induced AD mouse model, we demonstrated

Fig. 5. Total IgE Levels in Serum

Total IgE levels were measured by ELISA on days 0, 6, 13, 20, and 24 (○, vehicle; ●, TNCB; △ and ▼, TNCB+SeMet at 5 and 10 $\mu\text{mol/kg/d}$, respectively). The results are presented as mean $\pm$ S.D. ($n=6$). ** $p<0.01$, * $p<0.05$, compared with the TNCB-only group. Experiments were repeated independently at least three times and similar results were obtained.

Fig. 6. Effects of SeMet on Expression of IL-4 mRNA in Ears and Superficial Parotid Lymph Nodes

Ear (A) and superficial parotid lymph node (B) samples were collected 24h after the final challenge on day 24, mRNA was extracted, and IL-4 mRNA levels were evaluated using real-time quantitative PCR. The results are presented as mean $\pm$ S.D. ($n=6$). ** $p<0.01$, * $p<0.05$, compared with the TNCB-only group. Experiments were repeated independently at least three times and similar results were obtained.

that SeMet supplementation suppresses AD-like skin lesions, and inhibit IgE and IL-4.

Patients with allergic disease, such as AD and asthma,

Fig. 7. Effects of SeMet on Production of IL-4 in Ears and Superficial Parotid Lymph Nodes

Ear (A) and superficial parotid lymph node (B) were excised 24h after the final challenge on day 24, homogenized, and subjected to ELISA. The results are presented as mean $\pm$ S.D. ($n=6$). ** $p<0.01$, * $p<0.05$, compared with the TNCB-only group. Experiments were repeated independently at least three times and similar results were obtained.

showed significantly lower selenium concentration in the blood or nails.^{16–21} Thus, we speculated that supplementation of selenium compounds might be effective in preventing AD. However, the effects of selenium supplementation on AD remain unknown. In this study, we examined the effects of SeMet on AD-like skin lesions in BALB/c mice.

First, we confirmed that the reaction of the early- and delayed-type responses was caused by repeated application of TNCB. Ear thickness increased with repeated application of TNCB, resulting in a shift in the time course of ear thickness response from a typical delayed-type hypersensitivity response (peak at 24h after challenge) to an early-type response (peak at 6h), which is consistent with the results of a previous report.²⁴ SeMet supplementation suppressed the increase in the ear thickness at 6 and 24h after TNCB application in a dose-dependent manner. These results demonstrated that SeMet suppresses the early- and delayed-type responses in AD.

In this study, SeMet was orally administered to mice on days 0 to 23. The intake of selenium from diet was 1.0 $\mu\text{g}/\text{mouse/d}$ on average (data not shown). The dose of selenium administered to mice by SeMet administration was 7.4 $\mu\text{g}/\text{mouse/d}$ (5 $\mu\text{mol/kg/d}$) or 14.9 $\mu\text{g}/\text{mouse/d}$ (10 $\mu\text{mol/kg/d}$)

on average. It has been reported that oral administration of SeMet at a dose of 80.5 μg selenium/mouse/d for 28d is safe and nontoxic.³⁴⁾ Therefore, the dose used in this study is considered safe and non-toxic for mice. As we have previously reported, the increase in selenium concentration in the liver by SeMet administration is correlated with the increase in selenium concentration in other tissues, such as skin and blood.^{35,36)} Because other tissues and blood were reserved for real-time quantitative PCR or ELISA, we measured the selenium concentration in the liver as a representative. In this study, the selenium concentration in the liver increased following SeMet supplementation in a dose-dependent manner, and there was no significant difference in body weight, coat appearance, and general grooming between the SeMet-supplemented and control groups (data not shown).

Histological evaluation with hematoxylin and eosin staining showed that supplementation of SeMet inhibited epidermal hyperplasia and dense infiltration of inflammatory cells. Moreover, toluidine blue staining showed increased numbers of mast cells in the dermis after repeated TNCB application and this increase was suppressed by SeMet supplementation. Accumulations of mast cells have been associated with several cutaneous diseases, including AD. Natsuaki *et al.*³⁷⁾ reported that a local increase in mast cells by repeated application of a hapten is involved in early-type responses induced by repeated hapten application in mice. Therefore, inhibition of increased mast cell number in the ear due to SeMet supplementation is considered to be involved in the suppression of ear thickness by SeMet, especially in the early-type response.

Total IgE level was increased by repeated TNCB application, and this increase was suppressed significantly by SeMet. Furthermore, increased IgE on day 13 was gradually reduced in the SeMet supplementation group. IgE causes not only immediate-type reactions, but also chronic phase skin inflammations, such as AD, suggesting that the upregulation of total serum IgE may be a hallmark of AD.³⁸⁾ Therefore, the decreased IgE level was suggested to contribute to suppression of ear thickness by SeMet.

In our study, the expression of IL-4 mRNA and the production of IL-4 in the ear and superficial parotid lymph node on day 24 increased following repeated TNCB application, and these increases were inhibited by SeMet supplementation. IL-4 is produced in the skin of AD patients and is considered to play an important role in the pathogenesis of atopic dermatitis.^{4–6)} IL-4 is effective in stimulating the expression of IgE.³⁸⁾ These studies indicate that the reduction of IL-4 by SeMet is also involved in the suppression of AD. Repeated application of TNCB has been reported to induce Th2-polarized inflammation,^{28,39)} and the production of IL-4 in the regional lymph node involves Th2 cell development,^{40–43)} thus suggesting that SeMet might be involved in the differentiation of Th2 cells.

We concluded that SeMet supplementation suppresses AD-like skin lesions and inhibits expression of total IgE and IL-4.

Acknowledgments The authors thank Mr. Ryuma Nakahira and Mr. Takuma Hirooka for excellent technical assistance. This research was supported in part by Grant-in-Aid for Young Scientists (B) (JP15K18912) from the Japan Society for the Promotion of Science.

Conflict of Interest The authors declare no conflict of interest.

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