

A Novel Benzoimidazole Derivative, M50367, Modulates Helper T Type I/II Responses in Atopic Dermatitis Mice and Intradermal Melanoma-Bearing Mice

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The existence of helper-T cell (Th) subsets, types I and II (Th1/Th2), provides a framework for understanding pathological immune responses. We previously reported that a benzoimidazole derivative, M50367, acted directly on naïve Th cells to inhibit their differentiation into Th2 cells. Oral treatment with this compound reduced the Th2 response *in vivo* and suppressed disease progression in a murine model of atopic asthma. In this study, we investigated the effect of M50367 on 2 other murine disease models, such as atopic dermatitis and intradermal tumor-bearing mice, the pathogenesis of which may be related to the Th2 response. NC/Nga mice treated with a repeated application of picryl chloride developed atopic dermatitis-like skin lesions together with IgE hyper-production. M50367 (30 mg/kg) significantly inhibited the IgE hyper-production without affecting the skin lesions. In C57BL/6 mice bearing intradermal B16F10 melanoma, M50367 (30, 100 mg/kg) significantly inhibited splenomegaly and enhanced spontaneous interferon- γ release from cultured splenocytes in a dose-dependent manner, though its effect on tumor volume was limited. These results suggest that M50367 could reduce the Th2 response (IgE hyper-production) and enhance the Th1 response (splenocytes interferon- γ production) in these models. In contrast to previous results in the asthma model, its immunomodulation did not lead to the suppression of disease progression, indicating that the pathogenesis of these models might not simply depend on Th2 response.

Key words atopic dermatitis; tumor; helper T lymphocyte; IgE; interferon- γ

On the basis of their cytokine production profiles, CD4⁺ helper T lymphocytes (Th) were subdivided into two distinct populations, Th1 and Th2 cells. Th1 cells producing interferon- γ (IFN- γ) play a critical role in cellular immunity, while Th2 cells producing interleukin (IL)-4 and IL-5 are essential for the regulation of humoral immunity.^{1–3)} The balance between Th1- and Th2-dominant immunity (Th1/Th2 balance) was thought to be important for the development and maintenance of various diseases. For example, the predominance of Th2 and increased serum IgE level were reported in patients with atopic dermatitis^{4–6)}; in cancer patients, imbalance of Th1 and Th2 was found in the peripheral lymphocytes.^{7,8)} Thus, immunomodulators which can skew the Th1/Th2 balance into Th1 dominance may be useful drugs for treating atopic dermatitis and cancer.

Regarding the laboratory investigations on the effect of Th1/Th2 balance modulators such as IFN- γ and IL-12 (an inducer of IFN- γ) in atopic dermatitis and intradermal cancer models, there are several studies using NC/Nga (NC) mice^{9,10)} and intradermal transplantation of B16F10 melanoma.^{11,12)}

NC mice, an inbred strain established from fancy Japanese mice, develop atopic dermatitis-like skin lesions under conventional care, or upon treatment with repeated challenge with picryl chloride,^{13–15)} and have thus been considered a useful model for human atopic dermatitis.¹⁶⁾ The elevation of plasma IgE level has been reported to correlate with the appearance of skin lesions in NC mice¹³⁾; Th2-specific chemokines and their receptors have been also reported to be highly expressed in the lesions of the NC mice.¹⁷⁾ Although these findings suggest the possible involvement of Th2 development in the pathogenesis of NC mice, the reported effects of Th1/Th2 modulators are not necessarily coincident: one report demonstrated that IFN- γ or IL-12 could reduce skin

lesions as well as decrease the elevation of plasma IgE level,¹⁰⁾ but another report demonstrated that neither IFN- γ or IL-12 was able to reduce them, or worsened them.⁹⁾ Thus, the involvement of Th2 development in the pathogenesis of NC mice remains unclear.

On the other hand, B16F10 melanoma-bearing mice are a frequently used animal model for the evaluation of anti-cancer agents. IFN- γ production of splenocytes from B16F10-bearing mice was smaller than that of normal mice,¹⁸⁾ indicating Th2 dominance in Th1/Th2 balance. In fact, repeated injections of IFN- γ or IL-12 could suppress the tumor growth in B16F10-bearing mice.^{11,12)} Thus, treatment with these Th1 cytokines alone is effective in this model. However, down-regulation of Th2 may act negatively on the host defense against cancer, because several reports have shown that not only Th1 but also Th2 are important for anti-cancer immunity.^{19–21)}

We had been searching for orally-active synthetic compounds which could down-regulate Th2 response, and found a unique immunomodulator, M50367 (ethyl 3-hydroxy-3-[2-(2-phenylethyl)benzoimidazol-4-yl]propanoate, Fig. 1). This compound directly acted on naïve Th cells to suppress their differentiation into Th2 cells *in vitro*; because the development of Th1/Th2 is reciprocally regulated, this compound resulted in the enhancement of Th1 cell differentiation.²²⁾

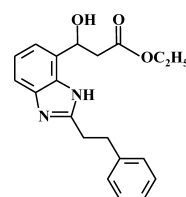


Fig. 1. Chemical Structures of M50367

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Oral treatment with M50367 reduced IL-4 production and enhanced IFN- γ production by splenocytes, indicating its ability to skew the Th1/Th2 balance into Th1 dominance, and it also suppressed disease progression, such as pulmonary eosinophilia and airway hyperreactivity in a murine model of atopic asthma.²³⁾ However, its efficacy on other disease models such as atopic dermatitis and tumor-bearing mice has not yet been elucidated.

In the present study, confirming the immunomodulating effect of M50367 on Th1/Th2 response in NC mice and B16F10-bearing mice by observing plasma IgE level or splenocyte IFN- γ production in these models, we evaluated the effect of M50367 on skin lesions or tumor growth of these models to clarify whether its immunomodulation of Th1/Th2 balance reflects the disease progression.

MATERIALS AND METHODS

Materials and Enzyme-Linked Immunosorbent Assay (ELISA) M50367 was synthesized in our laboratory. Hydroxypropylmethylcellulose (HPMC) was purchased from Sigma (MO, U.S.A.). Picryl chloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The IgE level in plasma samples was measured by a sandwich ELISA method as described by Hirano *et al.*²⁴⁾ The ELISA kit for interferon- γ (IFN- γ) was purchased from Endogen (Cambridge, MA, U.S.A.).

Mice Female NC mice aged 6 weeks were obtained from Clea Japan (Tokyo, Japan). Male C57BL/6 mice aged 6 weeks were obtained from Japan SLC (Shizuoka, Japan). All mice were kept in an air-conditioned and pathogen-free room at a temperature of $23 \pm 2^\circ\text{C}$ and humidity of $55 \pm 10\%$ on regulated 12 h light/dark cycle. They were given standard laboratory chow and tap water *ad libitum*. All experimental procedures mentioned below were in accordance with institutional guidelines for animal research.

Atopic Dermatitis Mice The dorsal skin and scalp were shaved from NC mice aged 7 weeks under anesthesia 3 d before the sensitization. These animals were sensitized by the application of 70 μl (both sides of right and left ears, 5 μl ; scalp, 10 μl ; dorsal skin, 40 μl) of 2% picryl chloride in acetone/ethanol (1:3) to shaved skin on day 0, and challenged by the application of 110 μl (both sides of right and left ears, 10 μl ; scalp, 20 μl ; dorsal skin, 50 μl) of 1% picryl chloride in olive oil on days 4, 7, 14, 21, 28, and 35.

Oral treatment with M50367 (30 mg/kg/d) was started on day 0 using 0.5% HPMC as a vehicle. A blood sample was taken once a week from the retro-orbital plexus for the measurement of plasma IgE by ELISA. Dermatitis symptoms were evaluated at 2, 4 and 6 weeks after the first picryl chloride application according to the scoring method described by Matsuda *et al.*¹³⁾ In brief, itching was evaluated by observing scratching behavior; edema was evaluated by measuring ear thickness; skin lesion was evaluated by macroscopic observation. Then, the dermatitis score was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for each of the five symptoms (itching, edema, hemorrhage, excoriation/erosion and scaling/dryness, total score=15).

Melanoma-Bearing Mice B16F10 melanoma-bearing mice were prepared as described previously.²⁵⁾ In brief, a

solid tumor was produced in male C57BL/6 mice aged 7 weeks by an intradermal injection, in the abdominal skin, of 0.1 ml of a cell suspension containing 10^6 cells. Tumor volume was calculated as $\text{length} \times \text{width}^2 \times 0.5$. After 7 d, animals bearing approximately 70 mm³ melanoma were treated with M50367 for 15 d using 0.5% HPMC as a vehicle. Twenty-two days after the tumor transplantation, each mouse was sacrificed to remove and weigh the thymus and spleen. From the spleen, splenocytes were prepared as described previously.²³⁾ The prepared splenocytes were then suspended in modified RPMI1640 (S-Clone SF-B, Sanko Junyaku Co., Ltd., Tokyo, Japan). One ml of the splenocytes suspension adjusted to 5×10^6 cells/ml was seeded in 48-well plates and cultured. After 24 h incubation at 37°C , the supernatant was harvested for ELISA.

Statistical Analysis Values are expressed as mean $\pm$ standard error of mean (S.E.M.). Results were evaluated by Dunnett's procedure for multiple comparisons or Wilcoxon's rank sum test using STAT LIGHT 1997 (Yukms Corp., Tokyo, Japan). The difference vs. vehicle-treated groups was considered to be significant when $p < 0.05$.

RESULTS

Atopic Dermatitis Model Figure 2 shows the onset or the progression of IgE hyperproduction and dermatitis in NC mice. The upper panel shows the change in plasma IgE level, which is considered an indicator of Th2 response.^{13,26)} Consistent with the previous report,¹⁴⁾ the plasma IgE level was gradually elevated by repeated challenges by picryl chloride. This elevation of IgE level was significantly ameliorated by the treatment with M50367, indicating its ability to down-regulate the Th2 response. The lower panel shows the dermatitis score. Also consistent with the same previous report,¹⁴⁾ repeated challenges by picryl chloride induced scratching behavior, ear thickness increase, and hemorrhage/excoriation/dryness of the skin. The dermatitis score

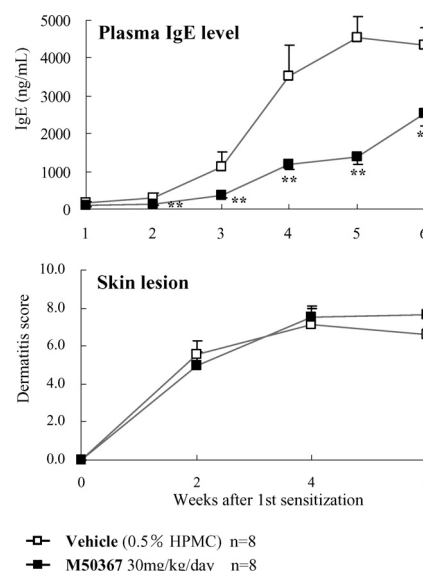


Fig. 2. Effect of M50367 on Plasma IgE Level (Upper Panel) and Dermatitis Score (Lower Panel) in NC Mice

Values are expressed as mean $\pm$ S.E.M. of 8 animals. ** $p < 0.01$ vs. vehicle-treated group calculated by Wilcoxon rank sum test.

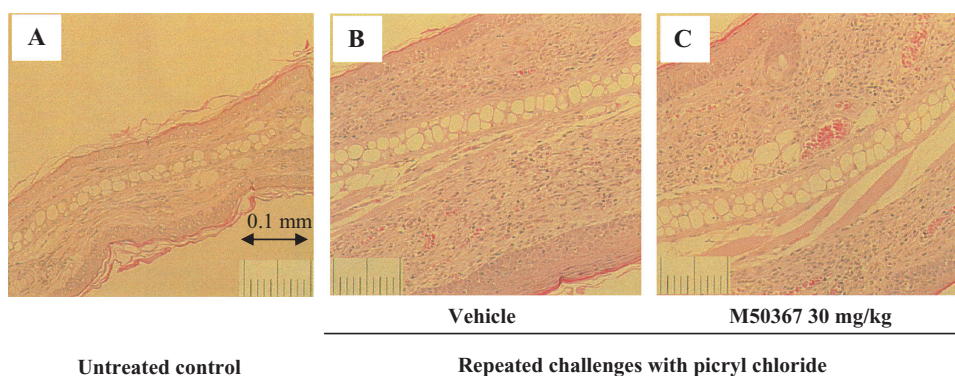


Fig. 3. Microscopic Observation of Hematoxyline and Eosin-Stained Ear Sections Derived from NC Mice

The ear was fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, stained and examined by light microscopy. A: NC mice not challenged with picryl chloride. B and C: NC mice treated with repeated challenges of picryl chloride.

evaluated by these symptoms increased within 2 weeks after the first challenge by picryl chloride, and the score was maintained at the same level by continuous challenges by picryl chloride. The change in dermatitis score was not affected by M50367 treatment.

Although M50367 had no effect on dermatitis in macroscopic observation, it might have some effect in microscopic observation. To check this possibility, histological analysis of ear skin was performed, and typical photographs are shown in Fig. 3. In comparison with untreated NC mice (Fig. 3A), repeated challenges by picryl chloride caused ear thickening by 3–4 fold, hyperplasia of the epidermis and dermis tissues, and massive infiltration of inflammatory cells, including eosinophils (Fig. 3B). These histological changes were not affected by M50367 treatment (Fig. 3C), indicating its lack of suppressive effect on dermatitis even in the microscopic observation.

Melanoma-Bearing Mice Figure 4 shows the immunomodulating effects of M50367 on C57BL/6 mice bearing B16F10 melanoma. In the tumor-bearing mice, thymus weight decreased by 0.5-fold (18 ± 3 mg) in comparison with that of normal mice (38 ± 6 mg). M50367 did not affect the thymus weight (upper panel) nor the body weight (vehicle treated group: 25.7 ± 0.8 g, M50367 30 mg/kg/d: 24.7 ± 1.2 g, and M50367 100 mg/kg/d: 25.4 ± 1.3 g). On the other hand, the spleen weight of tumor-bearing mice was 4-fold greater (222 ± 21 mg, middle panel) than that of normal mice (47 ± 6 mg). Such splenomegaly is a common pathological change in tumor-bearing states and may be related to the activation of humoral immunity (Th2 response). This splenomegaly was ameliorated by treatment with M50367 in a dose-dependent manner, and the change was statistically significant at 100 mg/kg/d, *p.o.* The lower panel of Fig. 4 indicates IFN- γ production by splenocytes, which may indicate the vigor of cellular immunity (Th1 response). IL-4 and IL-5 were undetectable in this experiment (data not shown). The IFN- γ production was augmented by the treatment with M50367 in a dose-dependent manner, and the change was statistically significant at 100 mg/kg/d, *p.o.*

Figure 5 shows the growth of intradermal B16F10 melanoma in C57BL/6 mice. Being similar to the several reports,^{12,25)} the animals could not survive because of the tumor growth on days 23–30 after the B16F10 transplantation in our preliminary experiment; no lung metastasis was seen,

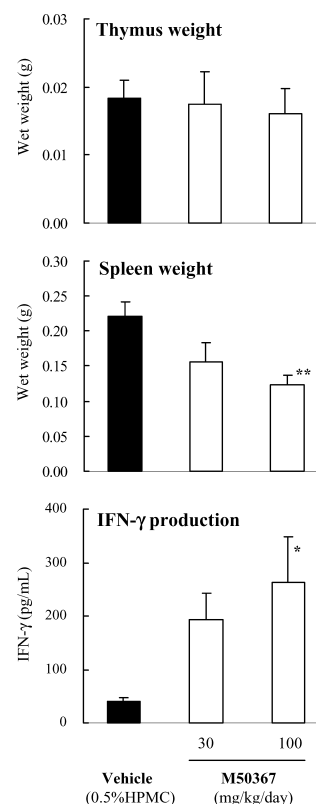


Fig. 4. Immunomodulating Effect of M50367 (30, 100 mg/kg/d) on Thymus Weight (Upper Panel), Spleen Weight (Middle Panel), and Spontaneous Interferon- γ Production by Splenocytes (Lower Panel) Derived from C57BL/6 Mice Bearing B16F10 Melanoma

Values are expressed as mean $\pm$ S.E.M. of 7 animals. **p* < 0.05 and ***p* < 0.01 calculated by Dunnett's procedure for multiple comparison.

and animals started to die of abdominal disruption and hemorrhage from the tumor in that period. In the present experiment, tumor volume also gradually increased until day 21; no animals died in any of the groups. The treatment with M50367 at 30 to 100 mg/kg/d tended to suppress the tumor volume on day 21, although the changes were not statistically significant, indicating the limited effect on tumor growth, nonetheless, of its immunomodulation.

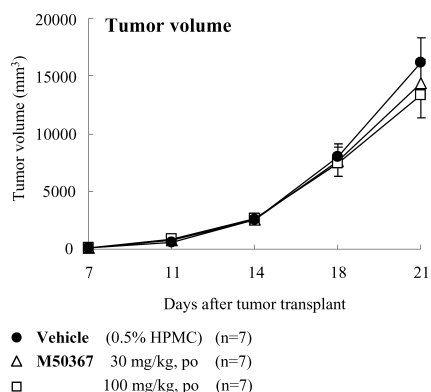


Fig. 5. Effect of M50367 on the Growth of Intradermal B16F10 Melanoma in C57BL/6 Mice

Tumor volumes were calculated as described in materials and methods. Values are expressed as mean $\pm$ S.E.M. of 7 animals. No statistically significant differences were observed among three experimental groups.

DISCUSSION

The aim of the present study was to clarify the effect of M50367 on Th1/Th2 response in atopic dermatitis and tumor-bearing mice, and to examine the relevance of the immunomodulation for disease progression in these models.

In NC mice, M50367 significantly reduced the IgE hyperproduction induced by the repeated challenge with hapten (picryl chloride). This result was similar to our previous results,²³⁾ in which M50367 significantly reduced the elevation of plasma IgE in BALB/c mice sensitized with allergens such as ovalbumin and parasite antigen. Since IgE production is an indicator of Th2 response, it is suggested that M50367 could down-regulate the Th2 response, even in NC mice, as well as in BALB/c mice. But contrary to our expectation, M50367 could not suppress the symptoms of atopic dermatitis or inflammatory cell infiltration into the skin of NC mice. Its lack of effect on disease progression was in contrast to our previous results in the asthma model²³⁾ in which M50367 suppressed symptoms of asthma (bronchial hyperactivity) and inflammatory cell infiltration in airways. These differences between the disease progression in atopic dermatitis and in asthma seemed to coincident well with the following reports with respect to signal transducers and activators of transcription (STAT) 6-deficient mice^{27–29)}: Th2 development and elevation of plasma IgE were completely suppressed in STAT6-deficient mice of any strains^{27–29)}; deletion of the STAT6 gene abrogated asthmatic bronchial hyperactivity and inflammatory cell infiltration in airways of allergen-induced asthma model,²⁹⁾ whereas the lack of STAT6 showed no effect on the symptoms of atopic dermatitis and inflammatory cell infiltration into the skin of NC mice.²⁷⁾ Considering that M50367 treatment, like STAT6-deficiency, could result in the inhibition of naïve Th differentiation into Th2 cells *in vitro*,^{22,28)} the reason M50367 could not suppress the dermatitis symptoms and inflammatory cell infiltration in spite of its reduction of IgE hyperproduction seemed to be based on the same cellular mechanism as that of STAT6 deficiency; therefore, we now conclude that the pathogenesis of skin lesions in NC mice might not be attributable to Th2 development.

In B16F10 melanoma-bearing mice, M50367 significantly

reduced splenomegaly and enhanced IFN- γ production by splenocytes. The reduction of splenomegaly by M50367 should not be due to its toxicity, because M50367 have been confirmed not to reduce the spleen weight of normal and allergen-sensitized mice in the previous report.²³⁾ The splenomegaly in tumor-bearing mice might indicate enhanced Th2-related humoral immunity, and its reduction by M50367 implicates the down-regulation of Th2 response. Similarly to our previous result using BALB/c mice,²³⁾ M50367 enhanced *ex vivo* IFN- γ production by splenocytes of the tumor-bearing C57BL/6 mice in the present study. Thus, M50367 was confirmed to be able to up-regulate the Th1 response, probably through the enhancement of Th1 differentiation even in C57BL/6 mice, as well as in BALB/c mice. In contrast, M50367 had little effect on tumor growth; the following report may provide a possible explanation for the reason M50367 could not effectively suppress tumor growth despite its enhancement of Th1 response: 1) STAT4-deficient mice lacking Th1 cells have comparative tumor immunity to wild type mice³⁰⁾; 2) STAT6-deficient mice lacking Th2 cells have enhanced tumor immunity, but Th1 cells do not seem to be involved because CD4 depletion does not diminish the anti-tumor effect,³⁰⁾ suggesting that enhanced tumor immunity by STAT6 deficiency may not depend on the Th1 development, but on macrophages and/or natural killer cells. Thus, the poor effect of M50367 on tumor growth may reflect that M50367 specifically act on naïve Th cell differentiation without affecting the functions of macrophages and/or natural killer cells.

In summary, M50367 skewed the balance of Th1/Th2 response in atopic dermatitis and tumor-bearing models similarly to the asthma model. But its immunomodulation did not lead to the suppression of disease progression, perhaps due to the independence of the Th1/Th2 response in these models. In addition to recent studies using genetic or cytokine-treatment approaches, the present data may provide another caveat to the hypothesis that manipulation of the Th1/Th2 response is a good strategy for the treatment of atopic dermatitis and tumor.

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