Effect of an Orally Active Th1/Th2 Balance Modulator, M50367, on IgE Production, Eosinophilia, and Airway Hyperresponsiveness in Mice

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Effect of an Orally Active Th1/Th2 Balance Modulator, M50367, on IgE Production, Eosinophilia, and Airway Hyperresponsiveness in Mice

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We have found a novel anti-allergic agent, M50367, which suppresses IgE biosynthesis and eosinophil accumulation in vivo. In this study, we evaluated the ability of M50367 to modulate Th1/Th2 balance in Th2-background BALB/c mice and to inhibit airway hyperresponsiveness in a murine model of atopic asthma. Oral M50367 at 3–30 mg/kg/day exhibited 51 to 73% reduction of IL-4/IL-5 production and 2–5-fold augmentation of IFN-γ production by Ag-stimulated cultured splenocytes of the mice sensitized with DNP-Ascaris. These alterations in Th1/Th2 cytokine production were accompanied by 55–85% suppression of plasma IgE level. Oral M50367 at a dose of 10 mg/kg/day significantly inhibited Ig-independent peritoneal eosinophilia by 54%, which was induced by repeated i.p. injections of Ascaris suum extract. To develop airway hyperresponsiveness caused by allergic airway inflammation, BALB/c mice were sensitized with i.p. OVA injections, followed three times by OVA inhalation. Oral M50367 significantly inhibited the increase in airway reactivity to acetylcholine, together with the elevation of plasma IgE level and pulmonary eosinophilia, which were observed in vehicle-treated mice 1 day after the last inhalation. Moreover, M50367 treatment reduced IL-4 and IL-5 production and tended to enhance IFN-γ production, not only by cultured splenocytes, but also in bronchoalveolar lavage fluid. These results suggest that M50367 has a modulating ability of Th1/Th2 balance to down-regulate Th2 response in the circulating system as well as at the sites of inflammation, and may be beneficial for the treatment of allergic disorders such as atopic asthma. The Journal of Immunology, 1999, 162: 7470–7479.

The existence of Th1/Th2 subsets in Th lymphocytes that differ in their cytokine secretion patterns and effector functions provides a framework for understanding normal and pathological immune responses (1). Allergic responses involving IgE-dependent mast cell degranulation and eosinophil accumulation in the sites of inflammation are considered to be due to the development and activation of Th2 cells (2). The representative cytokines of Th2 cells are IL-4 and IL-5. IL-4 is the major inducer of class-switching to IgE biosynthesis in B lymphocytes. IL-5 is the principal eosinophil-activating factor. On the other hand, IFN-γ, which is a representative cytokine of Th1 cells, is known to suppress the development of Th2 cells (3, 4). Since evidence suggested that the Th1 and Th2 types of reactions are reciprocally regulated in vivo (5–7), the modulation of Th1/Th2 balance, namely shifting the balance from Th2 to Th1 dominance, should be a rational strategy for the therapy of allergic diseases involving Th2 cells.

There are now numerous examples of experimental models in which modulation of the Th1/Th2 balance by administration of recombinant cytokines or cytokine antagonists (e.g., IL-12, IFN-γ, IL-4 antagonist, and IL-5 antagonist) alters the outcome of the diseases. For instance, IL-12 converts established Th2 response to Th1 dominance in some situations, suggesting its possible application in the treatment of allergy (8, 9). IFN-γ or Abs for IL-4 or IL-5 inhibit the pulmonary eosinophilia in allergic mice (10). However, the direct administration of such cytokines causes undesirable side effects and has limited their therapeutic use because of the lack of oral bioavailability and their cost. An alternative to cytokine therapy is to manipulate the endogenous Th1/Th2 balance by the administration of small molecules. For example, some non-peptide immunomodulators, such as a tellurium-based compound, AS-101 (11, 12), and streptococcal preparation, OK-432 (13), have activity to skew Th response toward Th1 dominance via the regulation of IL-10 or IL-12 production. However, they are not orally active. Thus, there have not yet been any orally active synthetic compounds that can modulate Th1/Th2 balance. If there were such compounds, they would play an important role in the treatment of allergic diseases to assist the therapy with glucocorticoids and blocking IgE- and eosinophil-mediated allergic inflammation. The putative indications of orally active Th1/Th2 modulators are considered to be asthma, allergic rhinitis, and atopic dermatitis. To the best of our knowledge, from recent basic and clinical reports, atopic asthma is one of the Th2-dominant diseases. Moreover, well-established experimental models of atopic asthma are now available. Therefore, we focused on this disease as the first indication of our new antiallergic agent.

We had been searching for the new compounds that suppressed the elevation of plasma IgE and found a novel synthetic compound, M50354 (3-[2-(2-phenylethyl)benzoimidazole-4-yl]-3-hydroxypropanoic acid) (see Fig. 1), which was i.v. and orally active but had lower oral bioavailability in rats (6%). After a slight molecular modification of M50354 in order to improve bioavailability, a new candidate of antiallergic drug, M50367 (see footnote 2) (Fig. 1, oral bioavailability in rats: 32%), was obtained. In this study, to evaluate the ability of M50367 to modulate the Th1/Th2 balance, we investigated its ex vivo effect on Th1/Th2 cytokine production by cultured splenocytes derived from Th2-background BALB/c mice. The effect of M50367 on allergic responses was

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2 Sample requests of these compounds, M50354 and M50367, (for scientific use), are available from Mr. Yutaka Kato at the address in footnote 1.
Cytokine levels in splenocyte supernatant were determined by sandwich ELISA kits. The kits for IL-2, IL-5, and IFN-γ were obtained from Endogen (Cambridge, MA), and IL-4 kits from PerSeptive Diagnostics (Framingham, MA). To check the viability of splenocytes, MTT assay was performed after 18 h of incubation according to the method described previously (14, 15). In brief, MTT dissolved in PBS at 5 mg/ml was added to the cultured splenocytes (final concentration: 0.5 mg/ml) and the culture plates were reincubated at 37°C for 4 h. The plates were then centrifuged and the supernatant was discarded. Extraction medium (SDS-Na 12.5 w/v%, N,N-dimethyl formamide 50 w/v%, pH 4.7) was added to all wells and the plates were shaken for 4 h with a plate shaker to dissolve unsolved dark crystals. After shaking, the plates were read on a Bio-Kinetic Reader EL312e (Bio-Tek Instruments, Winooski, VT) using a wavelength of 570 nm.

Mice sensitized with DNP-Ascaris. BALB/c mice were sensitized by an i.p. injection of 10 μg DNP-Ascaris adsorbed with 4 mg of alum on day 0. Then the sensitized mice were divided into eight groups (n = 6/group) and treated with 0.5% HPMC (vehicle, control mice); M50367, 3, 10, or 30 mg/kg; pranlukast (leukotriene antagonist), 30 or 100 mg/kg; or prednisolone, 3 or 10 mg/kg once a day from day 0 to day 9. Sham-sensitized mice (normal mice) were injected with saline and treated with vehicle. On day 10, after blood samples were taken from the retro-orbital plexus for the measurement of plasma IgG level, mice were sacrificed by cervical dislocation. Spleens were removed for measuring splenocyte cytokine production as described above. The splenocytes were cultured in the presence of 10 μg/ml DNP-Ascaris without any drugs. After these cultures were incubated for 18 h, the supernatant was harvested and cytokine levels were analyzed by ELISA. To check the effect of drugs on Ag-non-specific Th1/Th2 balance, a similar experiment was also performed using the splenocyte preparation in the presence of 5 μg/ml Con A in the absence of Ag. The viability of splenocytes were checked by MTT assay as described above. The total IgG level in plasma samples was measured by a sandwich ELISA method as described by Hirano et al. (16).

Direct effect on splenocyte Th1/Th2 cytokine production. This experimental protocol is essentially identical to the method for the evaluation of Th1/Th2 balance described above, except for the way of drug treatment. Briefly, mice were sensitized by an i.p. injection of 10 μg DNP-Ascaris adsorbed with 4 mg of alum. The sensitized mice were then kept for 10 days without any drug treatment. After 10 days of the sensitization, splenocytes were isolated from a pool of five spleens removed from these sensitized but untreated-mice. The splenocytes were suspended in the medium and seeded in 96-well culture plates at the cell density of 5 × 10^5 cells/ml in triplicate. They were cultured in the presence of 10 μg/ml DNP-Ascaris or 5 μg/ml Con A with 0.4–10 μg/ml drugs. M50354 (an active form of M50367), prednisolone, or cyclosporin A was added to the medium with 0.04% DMSO (vehicle). After these cultures were incubated for 18 h, the supernatant was harvested and cytokine levels were analyzed by ELISA. To examine the direct effect of drugs on cell viability, MTT assay was performed as described above.

To check cytokotoxicity of these drugs, their effects on cell viability of splenocytes and proliferative response to mitogens were assayed. The splenocytes of BALB/c mice were cultured in triplicate in 96-well plates at concentrations of 2 × 10^3 and 4 × 10^3 cells/0.2 ml/well, in the presence or absence of mitogen, respectively. Con A (0.5 μg/ml) was used as a T cell mitogen and LPS (20 μg/ml) was used as a B cell mitogen. Drugs were added as DMSO solutions to the culture at a final concentration of 0.1%.

The concentration of M50367, prednisolone, and cyclosporin A added in culture was 100 μM, 1 μM, and 1 μM, respectively. After 24-h incubation with modified RPMI 1640 medium, cell viability under no mitogen and proliferative response to Con A or LPS were measured by MTT assay.

Effect on allergic responses

Production of total IgE and Ag-specific Ig isotypes. BALB/c mice were sensitized with DNP-Ascaris as described above. Then the sensitized mice were divided into four groups and treated with 0.5% HPMC (vehicle, control mice) or M50367 (1, 3, or 10 mg/kg) once a day from day 0 to day 19. Blood samples were taken from the retro-orbital plexus on days 10 and 20. Total plasma IgE was measured as described above. DNP-Ascaris-specific Ig isotype levels were determined by sandwich ELISA in a 96-well plate according to the method described by Kuperman et al. (17) with slight modification. This method to measure Ag-specific IgG1 and IgG2a expression has been used to assess the relative influence of Th1 vs Th2 cytokines in vivo, since the production of IgG2a and IgG2b is augmented by IFN-γ and inhibited by IL-4, whereas the production of IgG1 is augmented by IL-4 and inhibited by IFN-γ (18). In brief, sample wells were coated with 10 μg/ml DNP-Ascaris in sodium bicarbonate solution (100 μl/well) and incubated at 4°C overnight. After three wash procedure with...
0.05% Tween-20 in PBS, wells were then blocked with 10% FBS in PBS (300 μl/well) at 37°C for 1 h. After washing, plasma samples were thawed and diluted with 10% FBS in PBS (1:5000 for IgG1, 1:100 for IgG2a, and 1:300 for IgG2b, respectively). Samples (100 μl/well) were incubated at 37°C for 1 h. Plates were washed and incubated with 100 μl/well biotin-conjugated anti-mouse Abs (1/5000 in yl chain-specific Ab, 1/2500 in y2a chain-specific Ab, and 1/2500 in y2b chain-specific Ab, respectively) at 37°C for 1 h. For the measurement of Ag-specific IgE, plasma samples diluted with 1:10 (100 μl/well) were applied to the plate coated with anti-mouse IgE Ab (1/1000, 100 μl/well), and incubated with 10 μg/ml biotin-conjugated DNP-Ascaris (100 μl/well). After another wash procedure, all plates were incubated with HRP-Streptavidin in 10% FBS at 37°C for 1 h. After a wash procedure with 150 μl/well citrate buffer, plates were developed with 1 mg/ml ABTS in citrate buffer containing 0.03% H₂O₂, then read at 405 nm within 30 min. Utilized dilution rate was settled to make OD values of all samples below the saturation point of the assay. Since recombinant DNP-Ascaris-specific Abs were not available to generate standard curves, relative Ab levels are calculated as % ΔOD values of control groups.

Ig-independent peritoneal eosinophilia. Murine peritoneal eosinophilia was produced by repeated stimulation with helminth Ag as previously described (19). Because the adoptive transfer of splenocytes, but not serum, from sensitized mice induces peritoneal eosinophilia (20), the pathway for eosinophil accumulation in this model is Ig independent. Mice were sensitized and challenged by i.p. injections of *Ascaris suum* extract (0.1 mg in saline) on day 0 and day 7. The sensitized mice were treated with 0.5% HPMC (vehicle), M50367, 1, 3, or 10 mg/kg, pranlukast (leukotriene antagonist) 30 or 100 mg/kg, or prednisolone 10 mg/kg once a day from day 0 to day 9. Sham-sensitized mice were treated with vehicle (normal mice). On day 10, after blood samples were taken from several mice to measure plasma total IgE (no IgE was detected), each mouse was sacrificed by decapitation and 3 ml of saline containing 1% EDTA-2K was injected into the peritoneal cavity. After the abdomen was massaged, peritoneal lavage fluid was recovered through a Teflon pipette. The peritoneal lavage fluid was then centrifuged at 200 × g for 10 min. The precipitate was resuspended in FCS containing 1% EDTA-2K. One hundred microliters of this cell suspension was dropped on slide glass, and stained by Microx Spiner HEG-NSP (Omron, Tokyo, Japan) to prepare a cell smear. The cell smear was stained with Diff-Quik (Kokusai Shiyaku, Tokyo, Japan) to determine the cellular component under a microscope. The remaining 50 μl of the suspension was diluted with 250 μl of saline containing 1% EDTA-2K and total cell number measured using a hemocytometer (Sysmex, Tochka Badens, Tokyo, Japan).

Murine model of atopic asthma

We have used a protocol slightly modified from that described by Nagai et al. (21). Mice aged 7 wk were actively sensitized by i.p. injections of 8 μg OVA and 80 μg Alum on day 0 and day 5. The sensitized mice were exposed either to OVA-saline (5 mg/ml) or saline aerosol for 30 min on day 10. Sham-sensitized mice were treated with vehicle. On the day of inhalation, prednisolone was administered about 1 h before, and M50367 was administered 30 or 100 mg/kg once a day from day 0 to day 9. Sham-sensitized mice were treated with vehicle (normal mice). On day 10, after blood samples were taken from several mice to measure plasma total IgE (no IgE was detected), each mouse was sacrificed by decapitation and 3 ml of saline containing 1% EDTA-2K was injected into the peritoneal cavity. After the abdomen was massaged, peritoneal lavage fluid was recovered through a Teflon pipette. The peritoneal lavage fluid was then centrifuged at 200 × g for 10 min. The precipitate was resuspended in FCS containing 1% EDTA-2K. One hundred microliters of this cell suspension was dropped on slide glass, and stained by Microx Spiner HEG-NSP (Omron, Tokyo, Japan) to prepare a cell smear. The cell smear was stained with Diff-Quik (Kokusai Shiyaku, Tokyo, Japan) to determine the cellular component under a microscope. The remaining 50 μl of the suspension was diluted with 250 μl of saline containing 1% EDTA-2K and total cell number measured using a hemocytometer (Sysmex, Tochka Badens, Tokyo, Japan).

Statistical analysis

Results were evaluated by one-way analysis of variance followed by Bonferroni’s procedure for multiple comparison. A difference among groups was considered to be significant when \( p < 0.05 \). Statistical analysis was conducted using STAT LIGHT 1997 (Yukms, Tokyo, Japan).

Results

Th1/Th2 balance in naïve mice

Fig. 2 shows the in vivo effect of M50367 on Th1/Th2 balance of naïve Th2 background mice. The cultured splenocytes derived from normal mice treated with vehicle secreted both Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-5) cytokines during 18-h incubation in the presence of Con A. In the mice orally treated with 10

![FIGURE 2. Th1/Th2 cytokine secretion by Con A-stimulated splenocytes from Th2 background mice treated with M50367. BALB/c mice were treated orally with M50367 for 10 days, then splenocytes were isolated from individual mice after 1 day of dosing and cultured in the presence of 1 μg/ml Con A without any drugs. The cell density was adjusted to 5 × 10⁵ cells/ml to obtain the uniformity of culture condition. Values are expressed as means ± SEM of five animals. *, \( p < 0.05 \); **, \( p < 0.01 \), calculated by Bonferroni’s procedure for multiple comparison.](http://www.jimmunol.org/)

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or 30 mg/kg M50367, the production of a representative Th1 cytokine, IFN-γ, by Con A-stimulated splenocytes increased, whereas the production of Th2 cytokines, IL-4 and IL-5, decreased in a dose-dependent manner, indicating that it skewed the Th1/Th2 balance of the mice toward Th1. The production of IL-2, which is another Th1 cytokine, was not changed by oral M50367 treatment. Neither change of cell viability nor the number of isolated splenocytes was seen in the groups treated with M50367, suggesting that it did not modulate cell proliferation but differentiation.

**Th1/Th2 balance in DNP-Ascaris-sensitized mice**

Fig. 3 shows the in vivo effect of drugs on Ag-stimulated Th1/Th2 cytokine production by cultured splenocytes and plasma IgE level of DNP-Ascaris-sensitized mice. The cultured splenocytes derived from the mice sensitized with DNP-Ascaris (control mice) secreted both Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-5) cytokines during 18-h incubation in the presence of the Ag, whereas the splenocytes from control-sensitized mice (normal mice) did not secret any of these cytokines. The plasma IgE level of control mice, which is an indicator of Th2 response, was elevated to reach 1.9 ± 0.2 μg/ml, whereas plasma IgE level of normal mice remained at a low level (0.06 ± 0.01 μg/ml). In the groups orally treated with 3 to 30 mg/kg M50367, the production of a representative Th1 cytokine, IFN-γ, by splenocytes increased by 2- to 5-fold, whereas the production of Th2 cytokines, IL-4 and IL-5, was 51 to 73% reduced in a dose-dependent manner. Well related 5-fold, whereas the production of Th2 cytokines, IL-4 and IL-5, was 51 to 73% reduced in a dose-dependent manner, indicating that it skewed the Th1/Th2 balance of the mice toward Th1. The production of IL-2, which is another Th1 cytokine, was not changed by oral M50367 treatment.

**Direct effect on splenocyte Th1/Th2 cytokine production**

Table I shows the direct effect of drugs on Th1 and Th2 cytokine production by DNP-Ascaris-stimulated splenocytes derived from the mice sensitized with the Ag. M50354, the active metabolite of M50367, had no direct action on cytokine production in vitro. It

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**FIGURE 3.** Th1/Th2 cytokine secretion by Ag-stimulated splenocytes derived from Ag-sensitized mice treated with M50367. BALB/c mice sensitized with DNP-Ascaris were treated orally with drugs for 10 days. After blood samples were taken to measure total IgE level, splenocytes were isolated from individual mice and cultured in the presence of 10 μg/ml DNP-Ascaris without any drugs. The cell density of all groups was adjusted to 5 × 10^6 cells/ml. Values are expressed as means ± SEM of six animals. *, p < 0.05; **, p < 0.01, calculated by Bonferroni’s procedure for multiple comparison.
had no effect when Con A was used instead of Ag, although the cytokine levels of the control group obtained by Con A stimulation were higher than those by Ag stimulation (data not shown). This result was in contrast with that of Fig. 3 in which M50367 showed the ability to skew the Th1/Th2 balance toward Th1 dominance in vivo, suggesting that this drug might have an activity to modify Th differentiation in vivo, but not have direct actions in vitro on differentiated Th1/Th2 cells. In contrast, prednisolone and cyclosporin A directly reduced both Th1 and Th2 cytokine production. These suppressive effects of these drugs on cytokine production were also observed in the case of Con A stimulation (data not shown). The effect of these immunosuppressants against cytokine production was accompanied with their complete reduction of the cytokine levels of the control group obtained by Con A or LPS and cell viability under no mitogen, but prednisolone reduced it to some extent.

Production of total IgE and Ag-specific Ig

Table II shows the change in plasma level of total IgE and Ag-specific Ig in DNP-Ascaris-sensitized mice treated with M50367. M50367 at doses of 1 to 10 mg/kg/day reduced both total and Ag-specific IgE in a dose-dependent manner. The extent of reduction was more prominent on day 20 than on day 10, indicating the advanced decrement of Th2 response. On day 10, M50367 significantly reduced the plasma level of Ag-specific IgG2a/IgG2b, which is an indicator of Th1 response, besides IgE. On the contrary, M50367 significantly reduced IgG1 and IgE levels, whereas no significant reduction was seen in IgG2a and IgG2b levels on day 20, indicating that the Th1 response tended to recover.

Peritoneal eosinophilia

Table III shows the effect of drugs on cell numbers (% of control) in peritoneal lavage fluid from the mice sensitized with Ascaris suum extract. The second Ag injection caused infiltration of lymphocytes, monocytes, and eosinophils into the peritoneal cavity of sensitized mice. Our preliminary results revealed that the plasma IgE level did not elevate before Ag challenge, and maximal eosinophil accumulation was observed after 3 days of Ag challenge. Neutrophils were scarcely seen on that day. The cell contents of the vehicle-treated group were as follows: lymphocytes are 71 ± 3%, monocytes 9 ± 1%, and eosinophils 19 ± 2%. As seen in Table III, M50367 at doses of 1 to 10 mg/kg/day inhibited the Ag-induced peritoneal eosinophilia in a dose-dependent manner. The inhibition rate (%) at the maximal dose of M50367 was 64%. Although M50367 slightly decreased the number of monocytes, the decrement was not so clear as in eosinophils. The glucocorticoid, prednisolone, at a dose of 10 mg/kg/day effectively inhibited the peritoneal eosinophilia by 78%. This drug had a tendency to decrease the numbers of lymphocytes and monocytes. In contrast, the leukotriene antagonist, pranlukast, at doses of 30 and 100 mg/
kg/day, had no effect on the cell accumulation of this model. Additionally, the loss of body weight was seen only in the mice treated with prednisolone. It was 23.9 ± 0.3 g and significantly lower than that of the control group (25.7 ± 0.4 g) on day 10.

**Murine model of atopic asthma**

Fig. 4 shows dose-response curves of bronchoconstriction caused by increasing doses of i.v. acetylcholine injections in OVA-sensitized BALB/c mice. OVA-inhaled mice in the control group clearly displayed airway hyperresponsiveness (AHR). The AHR in this experiment was characterized by a leftward shift in the dose-response curve of the control group compared with that of saline-inhaled group. M50367 at 30 mg/kg/day reversed the leftward shift to the right indicating the inhibition of AHR. Prednisolone at 10 mg/kg/day made the acetylcholine reactivity over-shifted when compared with the curve of the saline-inhaled group.

Fig. 5 shows the elevation of plasma IgE level and pulmonary eosinophilia assessed by counting the number of eosinophils in BALF in OVA-sensitized mice. As can be seen in Fig. 5, M50367 suppressed IgE production and pulmonary eosinophil infiltration in a dose-dependent manner, and these alterations were associated with the improvement of AHR at a dose of 30 mg/kg/day of M50367, which is shown in Fig. 4. Prednisolone at 10 mg/kg/day also significantly reduced the plasma IgE level and inhibited pulmonary eosinophilia in addition to the improvement of AHR.

Tables IV and V show cytokine levels in BALF and the supernatant of cultured splenocytes stimulated with OVA, respectively. OVA inhalation dramatically augmented IL-4 and IL-5 production, both in BALF and by cultured splenocytes. Th response in airways clearly skewed to Th2, because the IFN-γ level was low, but the IL-4 and IL-5 levels were high in BALF derived from control mice. The production of IFN-γ increased and those of Th2 cytokines, IL-4 and IL-5, decreased both in BALF and by the splenocytes from M50367-treated mice. The Th2 cytokine levels in BALF of M50367-treated mice tended to change according to the alterations observed in the splenocyte cytokine secretion. In contrast, the concentration of IFN-γ tended to decrease and that of IL-5 significantly decreased in the supernatant of cultured splenocytes derived from the mice treated with prednisolone. This simultaneous decrement of Th1 and Th2 cytokine secretion was related to the decrease of those cytokine levels in BALF derived from the

**FIGURE 4.** Airway reactivity to acetylcholine of OVA-sensitized mice. OVA or saline inhalation was performed three times at intervals of 3 days. One day after the last inhalation, mice were anesthetized and the increase in respiratory overflow volume provoked by acetylcholine was measured. Bronchoconstriction is represented as a percentage vs the maximal overflow volume. Values are expressed as means ± SEM. ***, p < 0.01, calculated by Bonferroni’s procedure for multiple comparison.

**FIGURE 5.** Plasma IgE level and pulmonary eosinophilia in OVA-sensitized mice. Values are expressed as mean ± SEM of 6–13 animals. *, p < 0.05; **, p < 0.01, calculated by Bonferroni’s procedure for multiple comparison.

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**Table III. Effect of M50367 on peritoneal eosinophilia induced by repeated i.p. injections of Ascaris suum extract**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg/day)</th>
<th>n</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
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</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>10</td>
<td>10</td>
<td>38 ± 4**</td>
<td>39 ± 8**</td>
<td>8 ± 2**</td>
</tr>
<tr>
<td>Sensitized mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1</td>
<td>5</td>
<td>100 ± 7</td>
<td>100 ± 13</td>
<td>100 ± 18</td>
</tr>
<tr>
<td>M50367</td>
<td>3</td>
<td>5</td>
<td>119 ± 14</td>
<td>84 ± 8</td>
<td>99 ± 8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>108 ± 13</td>
<td>74 ± 7</td>
<td>68 ± 23</td>
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<tr>
<td>Pranlukast</td>
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<td>4</td>
<td>89 ± 10</td>
<td>61 ± 7*</td>
<td>46 ± 8*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5</td>
<td>115 ± 11</td>
<td>67 ± 17</td>
<td>85 ± 13</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>10</td>
<td>5</td>
<td>141 ± 40</td>
<td>70 ± 9</td>
<td>107 ± 29</td>
</tr>
</tbody>
</table>

*Extract of Ascaris suum was injected into the peritoneal cavity of mice on days 0 and 7 to induce peritoneal eosinophilia. On day 10, peritoneal cells were harvested to count the number of eosinophils, as described in Materials and Methods. Drugs were orally administered from day 0 to day 9. Values (% of control) are expressed as means ± SEM.

* p < 0.05; ** p < 0.01 vs control by Bonferroni’s procedure for multiple comparison.
same group. Additionally, as seen in Table V, prednisolone dramatically decreased the total number of splenocytes. OVA inhalation increased the number of splenocytes per spleen by 40% when compared with that of the saline-inhaled group. This increment was abolished by the treatment with 30 mg/kg M50367. On the other hand, prednisolone treatment for 21 days overdecreased the number of splenocytes. It was under half of that of normal mice (2.6 ± 0.2 × 10^6 cells vs 6–8 × 10^6 cells).

Histological observations supported the anti-inflammatory action of drugs (Fig. 6). The Ag-induced airway inflammation in this experimental model was characterized by the eosinophil aggregation in the peribronchial area, edema in the lamina, goblet cell hyperplasia, and excessive secretion of PAS-positive mucus. These pathological alterations observed in the airway of OVA-inhaled control mice were ameliorated by M50367 treatment and completely diminished by prednisolone treatment.

The significant loss of body weight was observed in the group treated with prednisolone. On the other hand, no weight loss was seen in all groups treated with M50367. The body weight on day 21 of the normal group, the control group, the group treated with M50367 30 mg/kg/day, and the group treated with prednisolone (10 mg/kg/day) were 25.0 ± 0.1 g, 24.2 ± 0.4 g, 25.4 ± 0.3 g, and 22.8 ± 0.4 g (p < 0.05), respectively.

**Discussion**

This study demonstrates that oral treatment with a novel synthetic compound, M50367, modulates the pattern of splenocyte Th1/Th2 cytokine production in vivo, suppresses IgE biosynthesis, inhibits peritoneal eosinophilia, and is effective for Ag-induced airway hyperresponsiveness, as well as allergic pulmonary inflammation.

Th2-background BALB/c mice and Th1-background C57BL/6 mice are contrastive in their immune response (24, 25). The former strain develops allergic immune responses more easily than the latter strain because of its genetic backgrounds. In this study, M50367 treatment changed the pattern of Con A-stimulated Th1/Th2 cytokine production by splenocytes toward Th1-like phenotype in naive BALB/c mice. This result suggests that M50367 can skew the regular immune response of Th2 background mice toward Th1. Its activity to skew Th1/Th2 balance was more clearly observed when the mice were sensitized with Ag; the production of Th2 cytokines significantly decreased, whereas the production of IFN-γ was augmented in response to Ag or Con A stimulation. In contrast, M50354, the active form of M50367, showed no direct effect in vitro on both Th1 and Th2 cytokine production by splenocytes stimulated with Ag or Con A. These results suggest that M50367 has an activity to influence Th1/Th2 differentiation in vivo and does not directly influence Th1/Th2 cytokine production by established Th cells in vitro. The exact molecular mechanism by which M50367 skew Th response is still unknown at the present time. Since APC has a key role to Th differentiation (secretion of humoral factors, Ag presentation, and expression of costimulatory molecule), one possibility is that M50367 acts on APC. The other possibility is that it acts on Th progenitor cells. To elucidate these hypotheses, further studies are necessary; for example, in vitro naive Th progenitor differentiation system using OVA-specific αβ-TCR transgenic mice (26, 27).

This study revealed the effect of M50367 on allergic responses such as IgE production and eosinophilia. It effectively suppressed total IgE production in the mice sensitized with DNP-Ascaris or OVA. Although its reduction of Ag-specific Ig production was not

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### Table IV. Th1/Th2 cytokine levels in BALF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-5 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline inhalation</td>
<td>ND</td>
<td>7 ± 6**</td>
<td>4 ± 3**</td>
</tr>
<tr>
<td>OVA inhalation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>7 ± 3</td>
<td>45 ± 10</td>
<td>259 ± 31</td>
</tr>
<tr>
<td>M50367 (3 mg/kg/day)</td>
<td>11 ± 3</td>
<td>24 ± 5</td>
<td>166 ± 37</td>
</tr>
<tr>
<td>M50367 (10 mg/kg/day)</td>
<td>15 ± 6</td>
<td>14 ± 2**</td>
<td>136 ± 28**</td>
</tr>
<tr>
<td>Prednisolone (10 mg/kg/day)</td>
<td>ND</td>
<td>ND</td>
<td>17 ± 6**</td>
</tr>
</tbody>
</table>

* OVA-sensitized mice were exposed to OVA or saline aerosols three times. BALF were obtained from individual mice 1 day after the last aerosol inhalation. Values are expressed as means ± SEM of 6–13 animals.

* ND, not detected.

** p < 0.01 vs OVA-inhaled group treated with vehicle by Bonferroni’s procedure for multiple comparison.

### Table V. Th1/Th2 cytokine production by splenocytes stimulated with OVA and number of splenocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg/day)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-5 (pg/ml)</th>
<th>No. of Splenocytes (×10^7 cells/spleen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline inhalation</td>
<td></td>
<td>ND</td>
<td>136 ± 43**</td>
<td>7.7 ± 0.5**</td>
<td></td>
</tr>
<tr>
<td>OVA inhalation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>8969 ± 3695</td>
<td>17 ± 8</td>
<td>410 ± 69</td>
<td>10.6 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>M50367 (30 mg/kg/day)</td>
<td>4488 ± 2199</td>
<td>8 ± 4</td>
<td>64 ± 13**</td>
<td>7.4 ± 0.8**</td>
<td></td>
</tr>
<tr>
<td>Prednisolone (10 mg/kg/day)</td>
<td>14205 ± 9352</td>
<td>5 ± 3</td>
<td>56 ± 8**</td>
<td>2.6 ± 0.2**</td>
<td></td>
</tr>
</tbody>
</table>

* OVA-sensitized mice were exposed to OVA or saline aerosols three times. Splenocytes were isolated from individual mice 1 day after the last aerosol inhalation. The splenocytes were suspended at 5 × 10^6 cells/ml in the medium and seeded in culture plates in the presence of 1 mg/ml OVA. Values are expressed as means ± SEM of five animals.

* ND, not detected.

** p < 0.01 vs OVA-inhaled group treated with vehicle by Bonferroni’s procedure for multiple comparison.
fully specific to IgE on day 10, only Th2-mediated Ig classes (IgG1 and IgE) were significantly reduced by M50367 treatment on day 20, probably reflecting its down-regulation of Th2 response (Table II). However, it remains unknown why M50367 did not augment Th1-mediated Ig production (IgG2a and IgG2b) by increasing IFN-γ production. Concerning IgE biosynthesis by B cells, it is well known that IL-4 is the primal class-switching factor (28) and that the treatment with anti-IL-4 Ab reduces the plasma IgE level in the mice sensitized with OVA (29). The present study also revealed that the suppression of IgE production by M50367 was related to its suppression of the ability of splenocytes to produce IL-4 (Fig. 3). Prednisolone also suppressed IgE production in the OVA-sensitized mice (Fig. 5), but not in the DNP-Ascaris-sensitized mice (Fig. 3). Although the reasons for the difference are still unknown, it is noteworthy that the IgE suppression was accompanied by the suppression of IL-4 production by splenocytes in OVA-sensitized and OVA-inhaled mice, but not in DNP-Ascaris-sensitized mice. Therefore, M50367 and prednisolone suppressed IgE biosynthesis, probably by indirect or direct inhibition of IL-4 production by splenocytes.

Concerning eosinophilia, it is widely accepted that there are two different pathways for eosinophils to accumulate in tissues. One is T cells and their cytokine-dependent pathway, and the other is mast cells and the IgE-dependent pathway. In either pathway, IL-5, which is produced by T cells or mast cells, plays a principal role in controlling eosinophil proliferation, migration, activation, and survival in peripheral tissues (30, 31). In this study, M50367 inhibited eosinophil accumulation induced by i.p. injections of Ascaris suum extract. Previous studies have reported that T cells, but not mast cells binding IgE, are involved in the induction of peritoneal eosinophilia in this model (19, 20); the eosinophilia is observed only in nu/nu mice, but not in nu/nu mice, which do not have T cells (19), and adoptive transfer of splenocytes, but not serum, from sensitized animals induced the eosinophilia of recipients after the Ag challenge (20). These studies provide the evidence that T cells and their cytokines, like IL-5, are responsible for the eosinophil accumulation in this model. Since M50367 suppressed the IL-5 production by splenocytes in response to the Ag-stimulation in vivo, the inhibition of peritoneal eosinophilia exhibited by M50367 could be explained, at least in part, by its suppression of IL-5 production by T cells.

These activities of M50367 to suppress allergic responses such as IgE biosynthesis and eosinophil accumulation made us to expect clinical application of this compound as a new candidate of anti-allergic drugs. Because recent clinical reports have revealed that atopic asthma is one of the typical Th2-dominant diseases (32, 33), we tried to evaluate the efficacy of M50367 in a murine model of Ag-induced airway hyperresponsiveness (AHR) accompanied by

FIGURE 6. Photomicrographs of lung sections from mice sensitized with OVA. Lung tissues were fixed with phosphate-buffered formalin (10%) and embedded in paraffin. Longitudinal sections of 5 μm were stained with PAS reagent (×100). A, Normal aspect of lung from a sensitized mouse 24 h after the third inhalation of saline. B to E, The sensitized mouse 24 h after the third inhalation of 0.5% OVA in saline. B, Control: vehicle treatment. There was excessive PAS-positive material (mucus) in the lumen, aggregation of eosinophils in the peribronchial area, and damage of epithelial cells. These pathological changes in airway were ameliorated in C and D. C, M50367 treatment at a dose of 30 mg/kg/day. D, Prednisolone treatment at a dose of 10 mg/kg/day.
allergic airway inflammation. The sensitized mice treated with aerosolized Ag develop leukocytic infiltrates of the airway lumen dominated by Th lymphocytes and eosinophils. These mice also develop many of the pathological changes of atopic asthma, including AHR and goblet cell hyperplasia with excessive mucus production. Using the experimental protocol shown in this study, we too had reproduced such asthma-like histological changes and AHR. Th2 cell is reported to play an important role in airway eosinophil infiltration and/or AHR in response to Ag sensitization and challenge (34). Thus, the agents that might down-regulate Th2 responses would be expected to be effective in this model. Indeed, oral treatment with M50367 inhibited both airway eosinophil infiltration and AHR in the present study.

The pathway for the pulmonary eosinophilia in this model is likely to be dependent on IgE-FcɛR-mast cell axis, because previous reports have shown that the neutralization of IgE by anti-IgE Ab or blocking FcɛR by anti-CD23 Ab prevented eosinophil accumulation in the airway (35). Furthermore, recent works demonstrate that circulating IgE regulates FceRI expression on basophils and mast cells (36–38). Thus, the decrement in plasma IgE level, together with the decrement in FceRI expression, may reduce IgE-dependent airway mast cell degranulation followed by local IL-5 and/or chemokine production, which induces pulmonary eosinophilia. Therefore, the inhibition of pulmonary eosinophilia by M50367 could be explained by its prevention of mast cell activation via the suppression of IgE biosynthesis.

Another possibility was its suppression of IL-5 production by Th2 cells. In this study, the elevation of IL-5 levels in the supernatant of cultured splenocytes and in BALF were abolished by M50367 treatment. Thus, the inhibitory effect of M50367 on IL-5 production might be critical for the inhibition of pulmonary eosinophilia. In the meantime, AHR is a major clinical symptom of asthmatic patients. Many studies have revealed that Th2 cytokines play an important role in inflammatory pathways for the development of AHR in response to Ag sensitization and challenge. Nagai and his coworkers have reported that anti-IL-4 Ab treatment suppresses the elevation of IgE, and anti-IL-5 Ab treatment inhibits pulmonary eosinophilia, but either treatment alone cannot prevent the development of AHR in BALB/c mice (29, 39). On the other hand, treatment with both anti-IL-4 and anti-IL-5 Abs, which are expected to reduce IL-4 and IL-5 level, significantly improves AHR (40). In addition to these Abs, exogenous IFN-γ or mucus IFN-γ expression has also been reported to inhibit AHR (41, 42). Based on these reports, M50367 is considered to improve AHR by suppressing IL-4 and IL-5 production and by increasing IFN-γ production. However, this was not the case in prednisolone treatment, in which both Th2 cytokines and IFN-γ production were low level. In addition, its effect was accompanied by decrease in body weight and total number of splenocytes. The results in this study showing that prednisolone down-regulated both Th1 and Th2 responses were in good agreement with the report showing that it equally inhibits the proliferation and cytokine gene expression of both Th1 and Th2 clones (43). Thus, it is clear that M50367 improves AHR through a mechanism distinct from that of glucocorticoids. M50367 is therefore expected to become a safe alternative for the oral treatment with glucocorticoids without increasing risk of infections in consequence of the down-regulation of Th1-mediated host defense.

In summary, the pharmacological profiles of M50367 were as follows: the cytokine production pattern of splenocytes demonstrated that M50367 led to significant decrease in the Th2 response with concomitant increase in Th1 response, indicating systemic modulation of Th1/Th2 balance. These alterations have never been seen in mice treated with a glucocorticoid or a leukotriene antagonist. Moreover, the down-regulation of systemic Th2 response was reflected in the decrease in pulmonary local Th2 cytokine production and the suppression of IgE biosynthesis in mice exposed to aerosolized Ag. In this model of atopic asthma, M50367 effectively inhibited airway eosinophilic inflammation as well as AHR. Unlike prednisolone or cyclosporin A, M50367 had no cytotoxicity to splenocytes in vitro and no influence on body weight gain in vivo. In addition to the fact that it would be expected to not reduce Th1-mediated host defense, M50367 seemed to be safer than these immunosuppressants. To the best of our knowledge, M50367 is the first orally active Th1/Th2 balance modulator that may be beneficial for the therapy of allergic disorders such as atopic asthma.

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