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

Yutaka Kato, Tadashi Manabe, Yukiko Tanaka, and Hidenori Mochizuki

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- to 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 eosinophila 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 asthmatic disease 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, M503542 [3-[2-(2-phenylethyl)benzimidazole-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 antiallergy 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 available from Mr. Yutaka Kato at the address in footnote 1.

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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.

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also checked in some experimental models: in vivo IgE biosynthesis and Ig-independent peritoneal eosinophilia. Furthermore, considering atopic asthma as the first indication, we evaluated its effect on a murine model of Ag-induced airway hyperresponsiveness during allergic airway inflammation, and compared it with that of prednisolone.

Materials and Methods

Materials

Chemical compounds. M50354 and M50367 (ethyl ester of M50354) were synthesized and pranlukast (leukotriene antagonist) was extracted from commercial tablets in our laboratory. Prednisolone, cyclosporin A, gal-lamine triethiodide, and hydroxypropylmethylcellulose (HPMC)3 were purchased from Sigma (St. Louis, MO). Acetylichloride was obtained from Daichi Pharmaceutical Tokyo, Japan), sodium pentobarbital from Abbott Laboratories (Abbott Park, IL), and 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Wako Pure Chemical Industries (Osaka, Japan). All reagents used in this study were reagent grade.

Ag, mitogen, and Abs. OVA and Con A were purchased from Seikagaku Kogyo (Tokyo, Japan), LPS from Sigma, extract of Ascaris suum and DNP-Ascaris from Funakoshi (Tokyo, Japan), and HRP-Strepavidin from Zymed (San Francisco, CA). Biotin-labeled and unlabeled rat-anti-mouse IgE mAbs for measuring total and Ag-specific plasma IgE were obtained from Yamasa (Tokyo, Japan). Biotin-labeled anti-mouse IgG goat Abs (IgG1, IgG2a, and IgG2b) for measuring Ag-specific IgE were obtained from Southern Biotechnology Associates (Birmingham, AL).

Mice. Male BALB/c mice aged 6 wk were obtained from Japan SLCL (Shizuoka, Japan) and kept in an air-conditioned and pathogen-free room with temperature of 23 ± 2°C and humidity of 55 ± 10% on a regulated 12-h light/dark cycle. They were given standard laboratory chow (CE-2; Clea Japan, Tokyo) and tap water ad libitum, and acclimated at least 1 wk after receipt before use in all experiments. All experimental procedures mentioned below were in accordance with institutional guidelines for animal research. All drugs were suspended in 0.5% HPMC (vehicle) and given in a volume of 10 mg/kg to mice by gastric gavage.

Evaluation of Th1/Th2 balance

Naïve Th2 background mice. Unsensitized BALB/c mice were divided into three groups (n = 6/group) and orally treated with 0.5% HPMC (vehicle, normal mice) or M50367 (10 or 30 mg/kg) once a day from day 0 to day 9. On day 10, mice were sacrificed by decapitation and spleens were removed for measuring splenocyte cytokine production as described above. The mice were treated with 0.5 mg/ml DNP-Ascaris without any drugs. After these cultures were incubated for 18 h, the supernatant was harvested by centrifugation 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 same splenocyte preparation in the presence of 5 μg/ml Con A as a mitogen of Ag. The viability of splenocytes were checked by MTT assay as described above. The total IgE 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 and untreated-mice. The mice were treated with 0.5 mg/ml Con A and graded into two groups (vehicle, control mice) 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-oralibel plexus for the measurement of plasma IgE level, mice were sacrificed by decapitation and spleens were removed for measuring splenocyte cytokine production as described above. The mice were treated with 5 μg/ml DNP-Ascaris without any drugs. After these cultures were incubated for 18 h, the supernatant was harvested by centrifugation 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 same splenocyte preparation in the presence of 5 μg/ml Con A as a mitogen of Ag. The viability of splenocytes were checked by MTT assay as described above. The total IgE level in plasma samples was measured by a sandwich ELISA method as described by Hirano et al. (16).

Effect on allergic responses

Production of total IgE and Ag-specific Ig isotypes. BALB/c mice were sensitized with DNP-Ascaris as described above. 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-oralibel 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 and IgE 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 (500/5000 in y1 chain-specific Ab, 1/2500 in y2a chain-specific Ab, and 1/25000 in y2b chain-specific Ab, respectively) at 37°C for 1 h. For the measurement of IgG-specific Ab, plasma samples diluted with 1:10 (100 μl/well) were applied to the plate coated with antiman1 or m2 Abs (1/1000, 100 μl/well), and incubated with 100 μg/ml biotin-conjugated DNP-Asc (100 μl/well). After another wash procedure, all plates were incubated with HRP-Streptavidin in 10% FBS in PBS (100 μl/well, 1/4000) at 37°C for 1 h. After a wash procedure with 150 μl/well citrate buffer, plates were developed with 1 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-Asc-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 produces 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 0, 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 spun by Microx Spiner (Sysmex, 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, Tosa Kadenishi, 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 mixed with 100 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 12, 16, and day 20. The aerosol was generated with Ultrasonic Nebulizer NE-U12 (Omron, Tokyo, Japan). The mice were divided into eight groups after the first sensitization with OVA on day 0. Each group was treated with 0.5% HPMC (vehicle), M50367, 1, 3, or 10 mg/kg; or prednisolone 10 mg/kg once a day from day 12, day 16, and day 20. The OVA-sensitized and saline-inhaled mice were treated with vehicle. On the day of inhalation, prednisolone was administered about 1 h before, and M50367 was administered 10 to 30 min before, the inhalation. On day 21, about 24 h after the last inhalation and drug administration, blood samples were taken from the retro-orbital plexus and centrifuged to obtain plasma. The plasma IgE level was then measured as described above.

After blood sampling, the mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and the jugular vein was cannulated for i.v. injections of acetylsalicylic acid. The mice were ventilated with a rodent ventilator (SN-480-7, Shinnano Seisakusho, Tokyo, Japan) at 60 strokes/min with a stroke volume of 0.7 ml/body and pretreated with 20 mg/kg, i.v. gallamine triethiodide to avoid a signal noise of spontaneous respiration. Bronchoconstriction was measured with an airway transducer connected to a tracheal cannula, according to the overflow method described by Konzett and Rössler (22). To evaluate the airway reactivity in response to acetylsalicylic acid challenge, the changes in the respiratory overflow volume were measured when 100 to 3000 μg/ml acetylsalicylic acid was injected. The increase in respiratory overflow volume provoked by acetylsalicylic acid was represented as a percentage vs the maximal overflow volume obtained by clamping the tracheal cannula.

After the measurement of airway reactivity, bronchoalveolar lavage fluid (BALF) was obtained from each mouse. The trachea was cannulated and the air lumen was washed four times with a total of 1 ml of saline containing 0.1% BSA. The washed fluid was pooled and centrifuged (220 × g) at 4°C for 10 min. The resultant supernatant was collected in 150 μl of PBS containing 1% EDTA. This cell suspension was used for determination of the number of eosinophils as described above.

Splenocytes of each mouse were isolated as described above, and suspended in modified RPMI 1640 medium (5 × 105/ml/well). One milliliter of the cell suspension was then seeded in a 48-well culture plate in the presence of 1 mg/ml of OVA. The cultures were incubated for 18 h and the supernatant was collected. Then the supernatant was stored at −80°C until the determination of cytokine concentration by ELISA, and the pellet was resuspended in 150 μl of FCS containing 1% EDTA. This cell suspension was used for determination of the number of eosinophils as described above.

For histological evaluation of pulmonary tissue, several mice were anesthetized with pentobarbital sodium 24 h after the last inflation. According to the procedure described by Kung et al. (23), the lungs were perfused in situ by passing a 24-gauge ball-tipped needle into the pulmonary artery through the right ventricle and flushing the pulmonary vasculature with 3 ml of ice-cold PBS. The lungs were then removed and fixed with 10% phosphate-buffered formalin. The left lobe of the lung was embedded in paraffin, sectioned at 5 μm thickness, and stained with periodic acid–Schiff (PAS) reagent.

**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 mg/kg M50367, the cytokine profile was significantly different from that of the vehicle-treated group. In particular, the IL-5/IL-4 ratio was suppressed in the M50367 group, indicating a shift toward a Th1 cytokine profile.
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 secrete 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 also seen when Con A was used instead of Ag (data not shown), although several other cytokine level obtained by Con A stimulation were higher than by Ag stimulation, probably because of the increase in the number of stimulated T cell clones. On the other hand, neither treatment with leukotriene antagonist (pranlukast) nor treatment with glucocorticoid (prednisolone) affected the pattern of cytokine production by splenocytes and the level of plasma IgE.

To assess the influence of drugs on whole spleen and splenocyte viability, the number of splenocytes was calculated and MTT assay was performed. In general, the number of splenocytes collected from the spleen of each mouse varied among samples. In this experiment, it was significantly decreased by 53% in the group treated with prednisolone (10 mg/kg/day). The exact numbers of splenocytes (×10^7 cells/spleen) in the control group, 30 mg/kg M50367, 100 mg/kg pranlukast, and 10 mg/kg prednisolone, were 7.1 ± 0.3, 6.7 ± 0.6, 5.9 ± 0.5, and 3.8 ± 0.2 (p < 0.05), respectively. Thus, the cell density of all samples was adjusted to an identical concentration, 5 × 10^6 cells/ml, to obtain the uniformity of culture condition. After being seeded at the identical concentration, the cell growth of splenocytes derived from control mice, assessed by MTT assay, was accelerated by the addition of the Ag when compared with that of normal mice. This Ag-driven splenocyte proliferation was seen in all sensitized groups, and the extent of proliferation was not changed by any drug treatment, including prednisolone. The OD at 570 nm in each group, which was an indicator of cell growth, was as follows: 0.43 ± 0.03 in normal mice, 0.75 ± 0.06 in control mice, 0.71 ± 0.03 in the group treated with 30 mg/kg M50367, 0.81 ± 0.05 in the group treated with 100 mg/kg pranlukast, and 0.75 ± 0.01 in the group treated with 10 mg/kg prednisolone. In brief, neither change of number of splenocytes nor that of cell viability was seen in the groups treated with M50367, which was different from the groups treated with prednisolone.

**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.
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 Ag-driven cell proliferation evaluated by MTT assay (data not shown). Moreover, in the experiment of mitogen-driven splenocyte proliferation or viability (data not shown), M50354 at 100 μM did not affect the proliferation of splenocytes stimulated with Con A or LPS and cell viability under no mitogen, whereas these immunosuppressants with 1 μM completely blocked the proliferative action caused by these mitogens; cyclosporin A did not affect 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 levels 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/

### Table I. Direct effect of M50354 (the active metabolite of M50367) on splenocyte Th1/Th2 cytokine production

<table>
<thead>
<tr>
<th>Treatment of Splenocytes</th>
<th>Concentration (μg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-5 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal splenocytes</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48 ± 23</td>
<td>86 ± 38</td>
<td>9 ± 4</td>
<td></td>
</tr>
<tr>
<td>Sensitized splenocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (0.04% DMSO)</td>
<td>1</td>
<td>1149 ± 272</td>
<td>1155 ± 315</td>
<td>196 ± 24</td>
<td>483 ± 32</td>
</tr>
<tr>
<td>M50354</td>
<td>3</td>
<td>1101 ± 336</td>
<td>953 ± 70</td>
<td>182 ± 9</td>
<td>508 ± 63</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1452 ± 253</td>
<td>1155 ± 206</td>
<td>185 ± 20</td>
<td>464 ± 59</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>1</td>
<td>ND</td>
<td>18 ± 29</td>
<td>94 ± 14</td>
<td>71 ± 28</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>0.4</td>
<td>ND</td>
<td>255 ± 72</td>
<td>128 ± 13</td>
<td>65 ± 15</td>
</tr>
</tbody>
</table>

<sup>a</sup> BALB/c mice were sensitized with DNP-Ascaris and maintained without any drug treatment. Ten days after the sensitization, splenocytes were obtained from a pool of five spleens and were cultured in triplicate at the cell density of 5 × 10<sup>6</sup> cells/ml in the presence of 10 μg/ml DNP-Ascaris. M50354 or immunosuppressants were added to the medium with Ag in vitro. Results show means ± SD for triplicate cultures taken from one representative experiment.

<sup>b</sup> ND, not detected.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg/day)</th>
<th>n</th>
<th>IgG2a (% ΔOD)</th>
<th>IgG2b (% ΔOD)</th>
<th>IgG1 (% ΔOD)</th>
<th>IgE (% ΔOD)</th>
<th>Total IgE (ng/ml)</th>
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<td>Day 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1</td>
<td>5</td>
<td>100 ± 4</td>
<td>100 ± 5</td>
<td>100 ± 5</td>
<td>100 ± 11</td>
<td>1316 ± 210</td>
</tr>
<tr>
<td>M50367</td>
<td>3</td>
<td>5</td>
<td>95 ± 6</td>
<td>106 ± 4</td>
<td>103 ± 3</td>
<td>94 ± 12</td>
<td>1064 ± 67</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>89 ± 4</td>
<td>95 ± 3</td>
<td>99 ± 2</td>
<td>72 ± 12</td>
<td>837 ± 74*</td>
</tr>
<tr>
<td>Day 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1</td>
<td>5</td>
<td>100 ± 4</td>
<td>100 ± 10</td>
<td>100 ± 3</td>
<td>100 ± 36</td>
<td>1581 ± 245</td>
</tr>
<tr>
<td>M50367</td>
<td>3</td>
<td>5</td>
<td>94 ± 13</td>
<td>126 ± 22</td>
<td>100 ± 3</td>
<td>74 ± 16</td>
<td>1127 ± 101</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>89 ± 6</td>
<td>94 ± 13</td>
<td>93 ± 2</td>
<td>68 ± 11</td>
<td>925 ± 77*</td>
</tr>
</tbody>
</table>

<sup>a</sup> DNP-Ascaris sensitized mice were treated with M50367 or vehicle for 20 days. Blood samples were taken from individual mice at 10 days and 20 days after the sensitization. Total IgE and Ag-specific Ig levels were analyzed by ELISA as described in Materials and Methods. Since recombinant DNP-Ascaris-specific Abs were not available, relative Ab levels are represented as % ΔOD values of control groups (vehicle-treated mice). Values are expressed as means ± SEM.

<sup>b</sup> * p < 0.05; ** p < 0.01 vs control by Bonferroni’s procedure for multiple comparison.
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

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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>Percentages of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitized mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>5</td>
<td>38 ± 4**</td>
</tr>
<tr>
<td>M50367</td>
<td>1</td>
<td>5</td>
<td>100 ± 7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>119 ± 14</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>108 ± 13</td>
</tr>
<tr>
<td>Pranlukast</td>
<td>30</td>
<td>4</td>
<td>89 ± 10</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>10</td>
<td>5</td>
<td>141 ± 40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></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.

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**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.
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 × 10^6 cells vs 6–8 × 10^7 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>Dose (mg/kg/day)</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>
<td></td>
</tr>
<tr>
<td>OVA inhalation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>3</td>
<td>11 ± 3</td>
<td>24 ± 5</td>
<td>166 ± 37</td>
</tr>
<tr>
<td>M50367</td>
<td>10</td>
<td>15 ± 6</td>
<td>14 ± 2**</td>
<td>136 ± 28**</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>10</td>
<td>17 ± 6</td>
<td>10 ± 3**</td>
<td>76 ± 17**</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>8969 ± 3695</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>4488 ± 2199</td>
<td>17 ± 8</td>
<td>410 ± 69</td>
<td>10.6 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>M50367</td>
<td>14205 ± 9352</td>
<td>8 ± 4</td>
<td>64 ± 13**</td>
<td>7.4 ± 0.8**</td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>3292 ± 3426</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/− 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...
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-FceRI-mast cell axis, because previous reports have shown that the neutralization of IgE by anti-IgE Ab or blocking FceRI 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 decrease 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 antag-onist. 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 cyto-toxicity 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.

Acknowledgments

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References


