

Complement C3a Regulates Late Asthmatic Response and Airway Hyperresponsiveness in Mice¹

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Allergic asthma is a chronic inflammatory disorder of the airways characterized by biphasic airway obstruction and airway hyperresponsiveness. In this study, we attempted to elucidate the contribution of the complement C3a to these asthmatic symptoms. BALB/c mice sensitized by i.p. injections of OVA plus alum were challenged with OVA intratracheally four times. The fourth challenge caused a biphasic asthmatic response peaking at 10 min and 3–4 h, as well as airway hyperresponsiveness to methacholine. Histological examination revealed increased expression of C3a receptors in the lung on the fourth challenge. Additionally, the C3 level in serum 4 h after the fourth challenge was significantly reduced compared with that before the challenge. When a C3a receptor antagonist, SB290157, was administered i.p. 30 min before the fourth challenge, the late-phase asthmatic response and airway hyperresponsiveness induced by the fourth challenge were significantly inhibited, although the early-phase response was not influenced. In bronchoalveolar lavage fluid, neutrophil infiltration 24 h after the fourth challenge was reduced by the treatment. On the other hand, SB290157 suppressed the increased expression of IL-1 β in the lung in this model, and the intratracheal administration of IL-1 β induced airway obstruction, airway hyperresponsiveness, and neutrophil infiltration in normal mice. These results illustrate that C3a is involved in the development of the late asthmatic response and airway hyperresponsiveness. The mechanism leading to the development of these symptoms may correlate with the recruitment of neutrophils and/or the production of IL-1 β induced by C3a. *The Journal of Immunology*, 2009, 183: 0000–0000.

Allergic asthma is a chronic inflammatory disorder of the airways characterized by reversible airway obstruction and airway hyperresponsiveness associated with elevated levels of allergen-specific IgE (1, 2). The reaction to an allergen can be divided into two phases, early and late (3–5). The early asthmatic response is characterized by acute obstruction of the airway beginning within minutes of exposure. The response has been considered to be related to mast cell degranulation followed by chemical mediator-induced narrowing of the airway (6). The late response begins 3–6 h after exposure, and the airway's narrowing is associated with the migration of neutrophils, eosinophils, and lymphocytes from the bloodstream into the lung parenchyma and airway epithelium (6–8). Furthermore, airway remodeling such as goblet cell hyperplasia and subepithelial fibrosis is a characteristic feature, especially in chronic asthma (9–13).

The complement C3a, a 78-aa peptide fragment derived from proteolytic cleavage of the complement protein C3, is a potent proinflammatory mediator that modulates leukocyte activation, smooth muscle contraction, and vascular permeability through its binding to the C3a receptor (14–17). In patients with allergic asthma, increased levels of C3a in bronchoalveolar lavage fluid

(BALF)³ have been described after challenges with an allergen (17–19), suggesting the activation of the complement pathway through an allergen-induced mechanism. On the other hand, the attenuation of airway hyperresponsiveness to methacholine (MCh) in a C3a receptor-deficient murine model of pulmonary allergy has been reported (17, 20, 21). However, the role of C3a in asthmatic symptoms has not been fully elucidated, especially in the biphasic asthmatic response.

Mice make excellent models with which to analyze mechanisms of inflammatory disease because of the many immunological tools available for this species. In asthma research using mice, most of the focus has been on airway hyperresponsiveness and lung eosinophilia as major physiological parameters, and early and late asthmatic responses have yet to be well studied. Recently, we developed a mouse model showing a biphasic asthmatic response (22).

In this study, we demonstrated the increased expression of C3a receptors during the induction of a late asthmatic response, and that inhibition of the receptor using a C3a receptor antagonist suppressed the Ag-induced late asthmatic response and airway hyperresponsiveness to MCh. Additionally, we found that the inhibition may be associated with a reduction in production of IL-1 β in the lung. Furthermore, we showed that C3a regulates the airway remodeling in this model. The results identify C3a as a potential therapeutic target in allergic asthma.

Materials and Methods

Animals

Male 7-wk-old BALB/c mice were obtained from Japan SLC. These mice were maintained in a temperature-controlled environment with free access

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³ Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; MCh, methacholine; PAS, periodic acid-Schiff; sRaw, specific airway resistance.

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to standard rodent chow and water. The first sensitization was started 1 wk after arrival of the mice.

All of the experimental procedures were approved by the Experimental Animal Research Committee at Kobe Pharmaceutical University.

Sensitization and challenge

BALB/c mice were sensitized by i.p. injection with OVA (grade V; Sigma-Aldrich) adsorbed to alum (Wako). OVA was used at a dose of 50 μ g adsorbed to 1 mg of alum/0.2 ml/animal on days 0 and 14. Nonsensitized mice were injected with 1 mg of alum/0.2 ml/animal on days 0 and 14. Both the sensitized and nonsensitized mice were challenged on days 28, 29, 30, and 35 under anesthesia with escain (Mylan Pharmaceuticals) with 1% OVA in a volume of 20 μ l by intratracheal administration as reported previously (23). Additionally, the sensitized mice were challenged on days 28, 29, and 30 with 1% OVA, and then with saline on day 35 (sensitized nonchallenged group).

On days 28, 29, 30, and 35, a C3a receptor antagonist, SB290157 (Calbiochem), was i.p. administered 30 min before each of the challenges (designated "multiple treatment"). In another group of mice, SB290157 was i.p. injected only once 30 min before the fourth challenge (designated "single treatment").

Measurement of airway resistance

To evaluate the degree of airway resistance, specific airway resistance (sRaw; $\text{cmH}_2\text{O} \times \text{ml}/(\text{ml}/\text{s})$) was measured in conscious mice before and 10 min to 5 h after the first and fourth challenges using a two-chambered, double-flow plethysmograph system (Pulmos-I; MIPs) according to the method of Pennock et al. (24).

Airway hyperresponsiveness to MCh

To estimate the hyperresponsiveness to MCh, sRaw was measured before and after the intratracheal instillation of a MCh solution. Briefly, 24 h after the fourth Ag challenge, 20 μ l of increasingly higher doses of MCh (3.125, 6.25, and 12.5 μ g/ml) in solution was consecutively applied to the trachea of the nonsensitized and sensitized mice at 30-min intervals. sRaw was measured 2 min after the instillation of each dose of MCh.

Analysis of cells recovered by BAL

To evaluate airway inflammation, we examined the accumulation of inflammatory cells in BALF. Animals were killed with diethyl ether. The trachea was cannulated, and the left bronchi were tied for histological examination. Then, the right air lumen was washed twice with 0.5 ml of HBSS. The recovered lavage fluid was centrifuged at $120 \times g$ for 5 min at 4°C. The cell pellet was suspended with a defined volume (200 μ l/sample) of HBSS. The total leukocyte count in the lavage fluid was determined by staining with Turk's solution. For differential cell counts, BAL cells were stained with Diff-Quik solution (International Reagents). A minimum of 300 cells were counted under a microscope and, based on their morphological criteria, classified as macrophages, lymphocytes, neutrophils, or eosinophils.

Histological analysis

The left lungs were fixed in 10% neutral-buffered formalin, then dissected, embedded in paraffin, and cut 4- μ m thick. Sections were stained with H&E, periodic acid-Schiff (PAS), and Masson's trichrome.

Immunohistochemistry (C3a receptor and IL-1 β) was performed with paraffin-embedded sections. Serial 4- μ m-thick sections of lung were mounted on glass slides, dewaxed, and rehydrated with PBS. Endogenous peroxidase was blocked with 3% H_2O_2 in water for 30 min. After the blocking of nonspecific binding with diluted normal rabbit serum in PBS for 20 min, the sections were incubated for 1 h at room temperature with a polyclonal Ab against the C3a receptor (SC-14624, goat IgG; Santa Cruz Biotechnology) or a polyclonal Ab against IL-1 β (SC-1251, goat IgG; Santa Cruz Biotechnology). The slides were developed using the Vectastain Elite ABC goat IgG kit and the diaminobenzidine substrate kit for peroxidase (Vector Laboratories). Counterstaining was done with Mayer's hematoxylin. As a negative control, goat IgG was used.

Scoring for each section was evaluated on scale of 0 to 4 with increments of 0.5 by a blinded observer for inflammation (H&E), goblet cell hyperplasia (PAS), subepithelial fibrosis (Masson's trichrome), and immunohistochemistry (C3a receptor and IL-1 β).

Mesurement of cytokines in BALF and C3 in serum

Levels of IL-4, IL-5, IL-13, and IL-1 β in BAL fluids were measured using quantitative colorimetric sandwich ELISA kits (R&D Systems). The level of C3 in serum was measured using mouse complement C3 ELISA kits (Kamiya Biochemical).

Changes in sRaw, airway responsiveness, and inflammatory cells in response to IL-1 β

IL-1 β (R&D Systems) in solution (10 and 100 ng/mouse) was applied to the normal mice. sRaw was measured before and 5, 10, 20, and 40 min after the instillation of each dose of IL-1 β . Airway hyperresponsiveness to MCh was measured 24 h after the intratracheal administration of IL-1 β . Additionally, we examined the accumulation of inflammatory cells in BALF 24 h after the instillation of IL-1 β .

Statistical analyses

Statistical analyses were performed with a one-way ANOVA. If significant differences were detected, individual group differences were determined by a Bonferroni-Dunn test. A p value of <0.05 was considered statistically significant.

Results

Time course of changes in sRaw after the Ag challenge

Fig. 1A shows the time course of the changes in sRaw after the first and fourth challenges in sensitized mice. The first challenge caused a swift elevation of sRaw that peaked at 10 min, followed by gradual diminution until 1 h after the challenge, with no obvious changes in sRaw observed at 2–5 h (Fig. 1Aa). In contrast, the fourth challenge, which was performed 5 days after the third challenge, induced a significant biphasic elevation of sRaw, peaking 10 min and 3 h later (Fig. 1Ab). In the nonsensitized-challenged and sensitized-nonchallenged groups, no obvious changes in sRaw were observed. Also, sRaw of the sensitized mice was not significantly changed by the second or third challenge (data not shown). These results were consistent with our previous findings (22).

Throughout the above experiment, no significant differences in sRaw before the challenges were found between the nonsensitized-challenged, sensitized-nonchallenged, and sensitized-challenged groups: the mean $\pm$ SEM of sRaw values before the fourth challenge was 2.571 ± 0.033 in the sensitized-challenged group, 2.553 ± 0.041 in the sensitized-nonchallenged group, and 2.526 ± 0.036 in the nonsensitized-challenged group.

Expression of C3a receptors

Although the level of C3a receptor expression in the sensitized group 24 h after the first challenge was similar to that in the nonsensitized group, it had increased by the time of the fourth challenge. This increase in expression was sustained for at least 24 h after the fourth challenge (Fig. 1Bc). The C3a receptor in the lung was found to be strongly expressed in inflammatory cells (Fig. 1Bb).

Effects of SB290157 on the biphasic asthmatic response and airway hyperresponsiveness

To test whether the biphasic asthmatic response in this model is inhibited by systemic treatment with a C3a receptor antagonist, we analyzed the effects of treatment with a single dose or multiple doses of SB290157. Fig. 2A shows the effect of SB290157 (0.3, 3, and 30 mg/kg), administered i.p. 30 min before the first, second, third, and fourth challenges, on the biphasic asthmatic response at the fourth challenge. The multiple treatments with SB290157 dose-dependently inhibited the late response. However, the early response was not suppressed. On the other hand, a single treatment with SB290157 (30 mg/kg) 30 min before the fourth challenge also inhibited the late phase, with the degree of inhibition being similar to that for the multiple treatments at 30 mg/kg (Fig. 2B).

Fig. 2C shows the effects of treatment with a single dose or multiple doses of SB290157 (30 mg/kg) on the development of airway hyperresponsiveness to MCh assessed 24 h after the fourth challenge. The development of airway hyperresponsiveness was

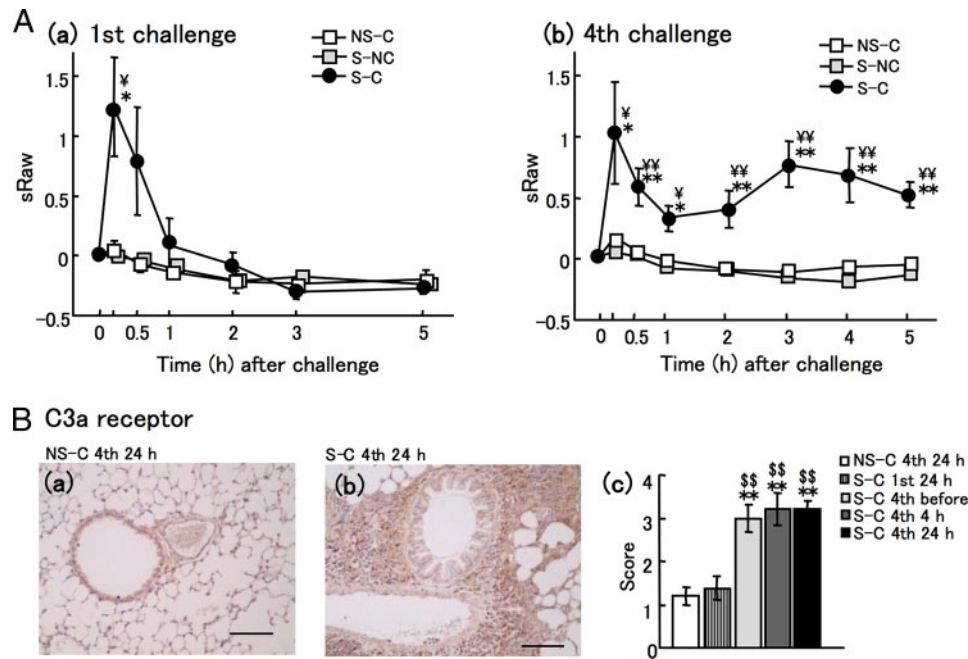


FIGURE 1. Time course of changes in sRaw and expression of the C3a receptor after the Ag challenges in sensitized mice. *A*, Changes in sRaw after the first (*a*) and fourth challenge (*b*) in nonsensitized-challenged (NS-C), sensitized-nonchallenged (S-NC), and sensitized-challenged (S-C) mice. *B*, Immunohistochemical localization of the C3a receptor in lung tissues 24 h after the fourth challenge in nonsensitized-challenged (*a*, NS-C 4th 24 h) and sensitized-challenged (*b*, S-C 4th 24 h) mice. Bar shows 100 μ m. Histological appearance 24 h after the fourth challenge in nonsensitized mice (NS-C 4th 24 h), 24 h after the first challenge in sensitized mice (S-C 1st 24 h), and 2 h before (S-C 4th before) or 4 (S-C 4th 4 h) and 24 h after the fourth challenge in sensitized mice (S-C 4th 24 h) was scored for the presence of the C3a receptor (*c*). Each value represents the mean $\pm$ SEM for six animals. *, $p < 0.05$ and **, $p < 0.01$ compared with the NS-C group. ¥, $p < 0.05$ and ¥¥, $p < 0.01$ compared with the S-NC group. \$\$, $p < 0.01$ compared with the S-C 1st group.

significantly inhibited by not only the single treatment but also multiple treatments.

Throughout the above experiments, no significant differences were found between sRaw values before and 24 h after the fourth challenge in each group (data not shown).

Time course of changes in C3 levels

Fig. 2*D* shows the C3 level in serum before, and 4 and 24 h after the fourth challenge in sensitized mice. The level at 4 h was significantly reduced compared with that before the fourth challenge in sensitized mice. The level was restored at 24 h after the challenge. This result indicates that C3 was activated to produce C3a during the late asthmatic response.

Effects of SB290157 on inflammatory cells in BALF

Fig. 3*A* shows the time course of changes in numbers of all leukocytes, macrophages, lymphocytes, neutrophils, and eosinophils in BALF after the fourth challenge. Numbers of leukocytes, macrophages, lymphocytes, and eosinophils had been increased before the fourth challenge and were not further increased 4 and 24 h after the challenge. On the other hand, although neutrophils were not detected before the fourth challenge, granulocytes were significantly increased 4 and 24 h after the Ag challenge.

The treatment with multiple doses of SB290157 significantly reduced numbers of all cells, macrophages, neutrophils, and eosinophils collected 24 h after the fourth challenge (Fig. 3*B*). On the other hand, the treatment with a single dose of SB290157 significantly inhibited neutrophils, but did not significantly affect increased numbers of macrophages, lymphocytes, and eosinophils (Fig. 3*B*).

Effects of SB290157 on cytokine production

The levels of IL-4, IL-5, and IL-13 twenty-four hours after the fourth challenge in sensitized mice were significantly increased,

although these cytokines were not detected in nonsensitized mice (Table I). The increased levels of IL-4, IL-5, and IL-13 detected 24 h after the fourth challenge in sensitized mice were not significantly reduced by treatment with either a single dose or multiple doses of SB290157 (Table I). On the other hand, IL-1 β levels increased only minimally 4 and 24 h after the fourth challenge in the sensitized mice (<5 pg/ml; data not shown).

Fig. 4 shows effects of a single dose or multiple doses of SB290157 on the production of IL-1 β in the lungs of sensitized mice. The level of IL-1 β before the fourth Ag challenge was similar to that in nonsensitized mice (Fig. 4, *a* and *b*), but significantly increased 24 h after the fourth challenge (Fig. 4*c*). Additionally, IL-1 β levels 4 h after the fourth challenge in sensitized mice were also significantly increased (data not shown). The increase in production of IL-1 β 24 h after the challenge was significantly suppressed by either a single treatment or multiple treatments with the C3a receptor antagonist (Fig. 4, *d* and *e*).

Changes in sRaw, airway responsiveness, and inflammatory cells induced by IL-1 β

Fig. 5 shows the changes in sRaw, airway responsiveness to MCh, and inflammatory cells induced by IL-1 β in mice. The intratracheal administration of IL-1 β dose-dependently increased sRaw, with a peak at 10 min after the instillation, and a decrease at 20 min (Fig. 5*A*). Additionally, IL-1 β induced airway hyperresponsiveness in a dose-dependent manner (Fig. 5*B*). Furthermore, there was an increase in leukocytes, especially neutrophils, 24 h after the intratracheal administration of IL-1 β (Fig. 5*C*).

Effects of SB290157 on inflammatory cells, goblet cell hyperplasia, and subepithelial fibrosis in the lung

As defined by the histological analysis using H&E staining, little leukocyte recruitment was observed 24 h after the fourth challenge in nonsensitized mice (Fig. 6*Aa*). In contrast, there was marked

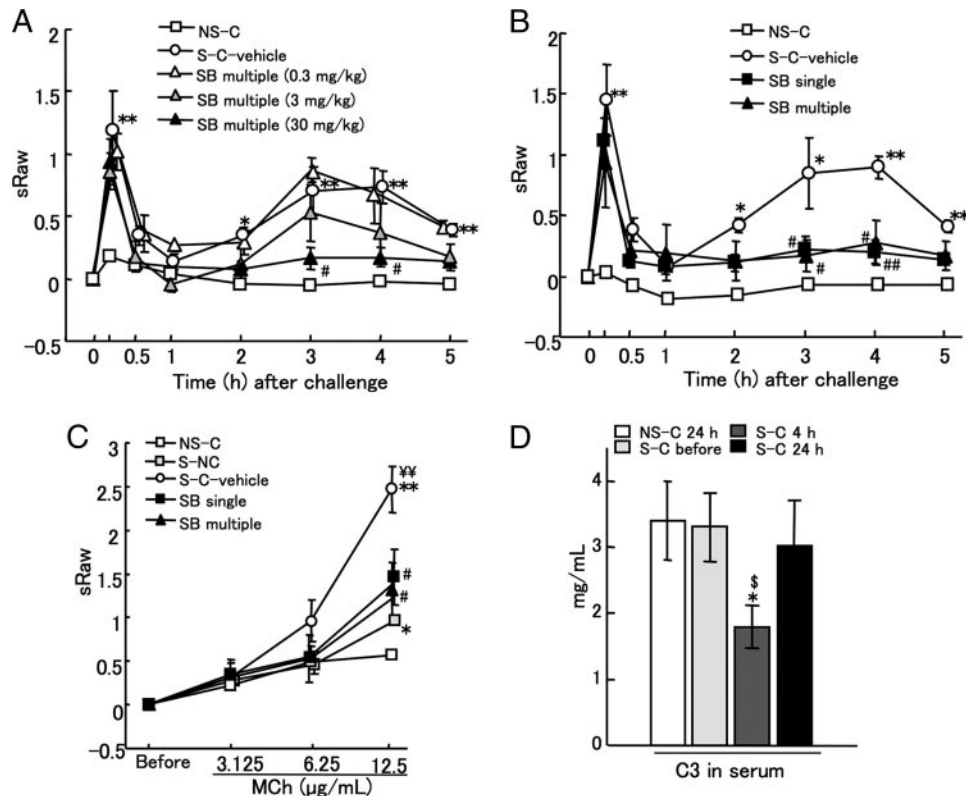


FIGURE 2. Effects of SB290157 (C3a receptor antagonist) on the biphasic asthmatic response and airway hyperresponsiveness after the fourth Ag challenge in sensitized mice. **A**, SB290157 at 0.3, 3, and 30 mg/kg was administered using a multiple dosing regimen (1 dose 30 min before each of the Ag challenges) in sensitized mice (SB multiple). The effects of the multiple treatments with SB290157 on the biphasic asthmatic response induced by the fourth Ag challenge were evaluated. Negative and positive controls were nonsensitized-challenged mice (NS-C) and sensitized-challenged, vehicle-treated mice (S-C-vehicle), respectively. **B**, The single treatment with SB290157 at 30 mg/kg in sensitized mice was administered 30 min before the fourth challenge in sensitized mice (SB single). The effects of treatment with a single dose or multiple doses of SB290157 at 30 mg/kg on the biphasic asthmatic response after the fourth challenge were examined. **C**, The effects of treatment with a single dose or multiple doses of SB290157 (30 mg/kg) on the development of airway hyperresponsiveness 24 h after the fourth challenge in sensitized mice. **D**, The C3 level in serum after the fourth Ag challenge in sensitized mice. C3 levels in serum 24 h after the fourth challenge in nonsensitized (NS-C) mice and 2 h before (S-C before) or 4 (S-C 4 h) and 24 h after the fourth challenge in sensitized (S-C 24 h) mice are shown. Each value represents the mean $\pm$ SEM for five to eight animals. *, $p < 0.05$ and **, $p < 0.01$ compared with the NS-C group. ¥, $p < 0.05$ and ¥¥, $p < 0.01$ compared with the S-NC group. #, $p < 0.05$ and ##, $p < 0.01$ compared with the S-C-vehicle group. \$, $p < 0.05$ compared with the S-C before group.

infiltration of leukocytes around the blood vessels and airways 24 h after the fourth challenge in sensitized mice (Fig. 6A*b*). Additionally, we performed a histological examination with PAS staining for the detection of goblet cells (Fig. 6B*b*) and with Masson's trichrome staining for the detection of fibrotic areas (Fig. 6C*c*). In the lungs of sensitized mice, both goblet cell hyperplasia (Fig. 6B*g*) and subepithelial fibrosis (Fig. 6C*i*) were observed 24 h after the fourth challenge. However, in the nonsensitized mice, goblet cell hyperplasia (Fig. 6B*f*) and subepithelial fibrosis (Fig. 6C*k*) were not observed. On the other hand, inflammatory cells, goblet cell hyperplasia, and subepithelial fibrosis had occurred in the lungs of sensitized mice by the fourth challenge and remained for at least 24 h (data not shown).

Fig. 6 shows the effects of treatment with a single dose or multiple doses of SB290157 on the histological changes in the lungs of sensitized mice. The multiple treatments with SB290157 significantly inhibited the infiltration of inflammatory cells (Fig. 6A*d*), goblet cell hyperplasia (Fig. 6B*i*), and subepithelial fibrosis (Fig. 6C*n*). In contrast, the single treatment did not produce any inhibitory effects.

Discussion

Consistent with our previous findings (22), the first administration of Ag induced an early asthmatic response without a late response,

but the fourth challenge caused not only an early but also a late asthmatic response in this model. Furthermore, airway hyperresponsiveness to MCh was observed 24 h after the fourth challenge. However, the mechanisms of the biphasic asthmatic response and airway hyperresponsiveness are not entirely clear. In this study, we focused on the role of C3a in these symptoms and found that the expression of C3a receptors was increased at the fourth challenge, but not the first challenge, in the sensitized mice. This increase in expression was correlated with the development of a late asthmatic response in sensitized mice. These findings prompted us to examine the effect of a specific C3a receptor antagonist, SB290157. Multiple treatments with SB290157 significantly suppressed both the late asthmatic response and hyperresponsiveness. These results clearly show the important roles of C3a signaling through C3a receptors in the development of a late asthmatic response and hyperresponsiveness.

Consistent with previous finding in a murine model of asthma (25), the expression of the C3a receptor was increased in epithelial cells and smooth muscle cells. Additionally, inflammatory cells in the lung also strongly expressed the C3a receptor in sensitized mice. These increases in expression had occurred by the time of the fourth challenge, suggesting that the first three challenges had produced sufficient inflammation of the airway to induce a late

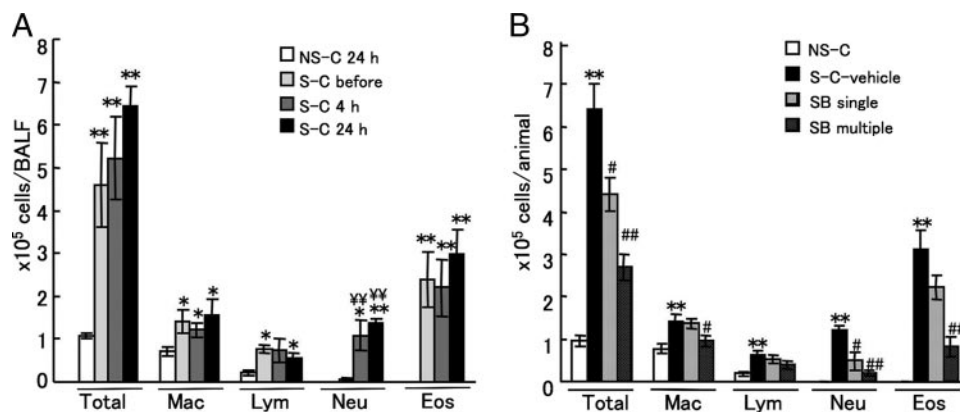


FIGURE 3. Effects of SB290157 (C3a receptor antagonist) on inflammatory cells in BALF after the fourth Ag challenge in sensitized mice. *A*, Data shown represent changes in inflammatory cells in BALF 24 h after the fourth challenge in nonsensitized (NS-C 24 h) mice and 2 h before (S-C before) or 4 (S-C 4 h) and 24 h after the fourth challenge in sensitized (S-C 24 h) mice. Each value represents the mean $\pm$ SEM for six animals. *B*, Data shown represent the effects of treatment with a single dose (SB single) or multiple doses (SB multiple) of SB290157 on the number of inflammatory cells in BALF 24 h after the fourth challenge in sensitized mice. Negative and positive controls were nonsensitized-challenged mice (NS-C) and sensitized-challenged, vehicle-treated mice (S-C-vehicle), respectively. Each value represents the mean $\pm$ SEM for five to eight animals. *, $p < 0.05$ and **, $p < 0.01$ compared with the NS-C group. ¥¥, $p < 0.01$ compared with the S-C before group. #, $p < 0.05$ and ##, $p < 0.01$ compared with the S-C-vehicle group. Total, all cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

asthmatic reaction in response to the fourth challenge. Additionally, as demonstrated in our previous report (22) and in the present study, inflammatory cells had increased in the lung before the fourth challenge. It has been reported that human C3a receptors were expressed on neutrophils, monocytes, and eosinophils in peripheral blood (26). Activated human T lymphocytes have been also reported to express C3a receptors (27). Thus, it can be speculated that the C3a receptor is expressed on inflammatory cells.

The concentration of C3a in BALF was elevated in patients having a late asthmatic response as compared with nonatopic control patients (17). On the other hand, in our model, the level of C3 in serum was reduced at 4 h (late phase) after the fourth administration of Ag in comparison with before the fourth challenge, and returned to the baseline level by 24 h, suggesting that the complement was activated during the late phase. These results imply that C3a is derived from the proteolytic cleavage of the complement protein C3 in the late phase in this model. However, the mechanism of C3a production in asthmatic mouse models is unclear. First, it is possible that in addition to activating the complement through the classical pathway, the Ag may directly activate C3 via an alternative pathway. Another possibility is that a neutral tryptase released from mast cells or pulmonary macrophages proteolytically cleaves C3 to generate C3a (28, 29). Thus, the Ag-triggered release of tryptase in sensitized mice could lead to C3a production without typical activation of the entire complement cascade. On the other hand, in this model, levels of Ag-specific IgE and IgG Abs in serum had already increased before the first challenge and

rose further before the fourth challenge (22). However, the multiple treatments with the C3a receptor antagonist did not have any inhibitory effect on the production of these Abs (data not shown). Therefore, the inhibition of the late phase response and airway hyperresponsiveness by the antagonist cannot be related to the production of these Abs.

Similar to the effect on the late asthmatic response and hyperresponsiveness, the multiple treatments with the C3a antagonist SB290157 inhibited the recruitment of inflammatory cells, including macrophages, neutrophils, and eosinophils in BALF. C3a produced during the four challenges played important roles in the recruitment of inflammatory cells into the lung. This finding was consistent with reports that C3a contributes to the accumulation of leukocytes such as macrophages, neutrophils, and eosinophils (30–33). On the other hand, SB290157 inhibited the late asthmatic response and hyperresponsiveness with only a single treatment before the fourth challenge, and inhibitory levels were similar to those in the multiple-treatment mice. Additionally, the single treatment significantly inhibited the recruitment of neutrophils, but not other cells, into the lung. In this model, the recruitment of neutrophils started 4–24 h after the fourth Ag challenge, although it was not observed before the fourth challenge. From these results, it was concluded that the recruitment of neutrophils during the late phase triggered the late asthmatic response and airway hyperresponsiveness in our model. Additionally, it has been reported that C3a can induce smooth muscle contraction and vascular permeability (14–17). Thus, the late asthmatic response may be induced directly by the contraction of smooth muscle and/or vascular permeability caused by C3a. On the other hand, although SB290157 has been reported to be a partial agonist of the C3a receptor in some systems in vitro (34), it has been used as an antagonist by several groups and has consistently shown the ability to act as an in vivo antagonist in a number of disease models through the attenuation of inflammatory responses (35–38). Therefore, its inhibitory efficacy in our model suggests the involvement of C3a in the late asthmatic response and airway hyperresponsiveness.

Although asthma has long been considered an eosinophilic bronchitis, its symptoms were not ameliorated by a marked anti-IL-5-induced reduction in eosinophil number in blood and the airway (39, 40). At the same time, the recognition that some asthmatics, particularly those who have severe asthma (41–43) and are

Table I. Effects of SB290157 (C3a receptor antagonist) on IL-4, IL-5, and IL-13 levels 24 h after the fourth Ag challenge in sensitized mice^a

	IL-4	IL-5	IL-13
NS-C	ND	ND	ND
S-C-vehicle	118 $\pm$ 36	20 $\pm$ 5	77 $\pm$ 15
SB single	91 $\pm$ 45	26 $\pm$ 10	79 $\pm$ 32
SB multiple	109 $\pm$ 45	15 $\pm$ 7	65 $\pm$ 23

^a Data represent the effects of treatment with a single dose (SB single) or multiple doses of SB290157 (SB multiple) on IL-4, IL-5, and IL-13 levels in BALF 24 h after the fourth challenge in sensitized mice. Values are in pg/ml. Negative and positive controls were nonsensitized-challenged mice (NS-C) and sensitized-challenged, vehicle-treated mice (S-C-vehicle), respectively. Each value represents the mean $\pm$ SEM for five to eight animals. ND, not detectable.

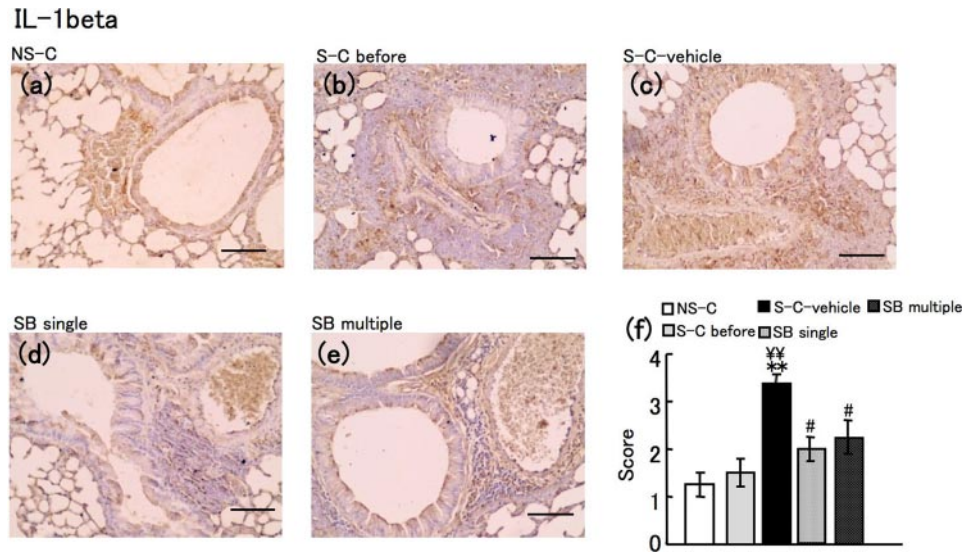


FIGURE 4. Effect of SB290157 (C3a receptor antagonist) on IL-1 β production after the fourth Ag challenge in sensitized mice. The immunohistochemical distribution of IL-1 β in lung tissue 24 h after the fourth challenge in nonsensitized (a, NS-C) mice and 2 h before (b, S-C before) and 24 h after the fourth challenge in sensitized (c, S-C-vehicle) mice is shown. Additionally, the effects of treatment with a single dose (d, SB single) or multiple doses (e, SB multiple) of SB290157 on the production of IL-1 β 24 h after the Ag challenge in sensitized mice were also evaluated. The bar shows 100 μ m. Histological appearance was scored for the presence of IL-1 β (f). Each value represents the mean $\pm$ SEM for five to eight animals. **, $p < 0.01$ compared with the NS-C group. ¥¥, $p < 0.01$ compared with the S-C before group. #, $p < 0.05$ compared with the S-C group.

resistant to corticosteroids (44), have raised neutrophil counts in their airways has led to suggestions that neutrophils may be a more valid target than eosinophils when attempting to bring asthma under control. Additionally, the fact that activated neutrophils release a large array of inflammatory mediators, oxygen radicals, and proteases has lent support to the notion of their involvement in the intense inflammation found in severe asthma (45, 46), suggesting that the increased numbers of neutrophils in the lungs are associated with asthmatic symptoms.

It is thought that IL-1 β is an important proinflammatory cytokine in asthma because an increase in its production was observed in asthmatic patients (47–49). Additionally, IL-1 β has been known to induce neutrophilic inflammation (50). Therefore, we examined whether the C3a receptor antagonist inhibits the production of IL-1 β in the lung. IL-1 β expression in the lung was increased after the fourth challenge in sensitized mice, and the increase was inhibited by either a single dose or multiple doses of the antagonist. Furthermore, the intratracheal administration of IL-1 β induced obstruction of the airway, the development of airway hyperresponsiveness to MCh, and the recruitment of neutrophils in BALF,

suggesting IL-1 β to be an important proinflammatory cytokine in the development of asthmatic symptoms. It has been established that inflammatory cells, for example macrophages (51–54) and epithelial cells (55), produce IL-1 β . In this case, C3a may cause IL-1 β to be produced by stimulating not only epithelial cells but also macrophages in the lung because macrophages had been recognized in the lung before the fourth challenge in the sensitized mice. Based on these results, IL-1 β produced in response to C3a in the lung may be associated with the development of the late asthmatic response and airway hyperresponsiveness.

Through the release of cytokines such as IL-4, IL-5, and IL-13, Th2 cells are thought to contribute to hyperresponsiveness and the hypersecretion of mucus as well as orchestrate the recruitment of inflammatory cells (56–59). Airway hyperresponsiveness and Th2-type cytokine production were diminished in C3a receptor-deficient mice (20, 21), suggesting that C3a plays an important role in these responses during the sensitization and/or effector phase. In our model, the Th2-type cytokines IL-4, IL-5, and IL-13 were clearly increased in BALF. Therefore, we examined whether the C3a receptor antagonist inhibits the production of cytokines at the

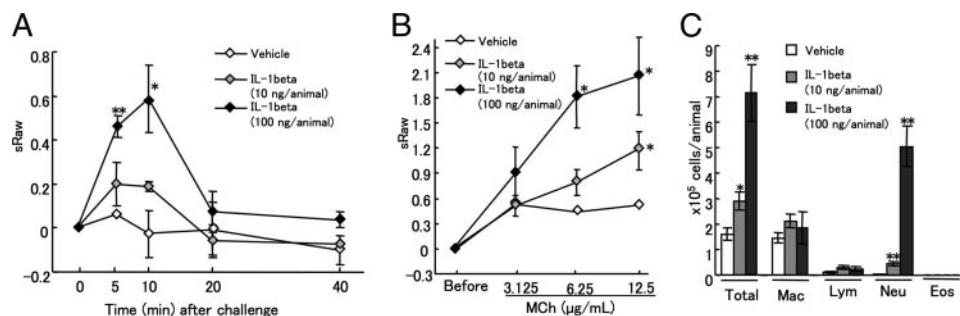


FIGURE 5. Changes in sRaw, airway responsiveness, and inflammatory cells caused by IL-1 β in mice. Data represent changes in sRaw (A), airway hyperresponsiveness to MCh (B), and inflammatory cells in BALF in IL-1 β -treated mice (C). IL-1 β (10 and 100 ng/animal) was intratracheally administered to normal mice. The measurements of airway responsiveness and collection of BALF were conducted 24 h after the instillation of IL-1 β in normal mice. Each value represents the mean $\pm$ SEM for five animals. *, $p < 0.05$ and **, $p < 0.01$ compared with the vehicle treated group. Total, all cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

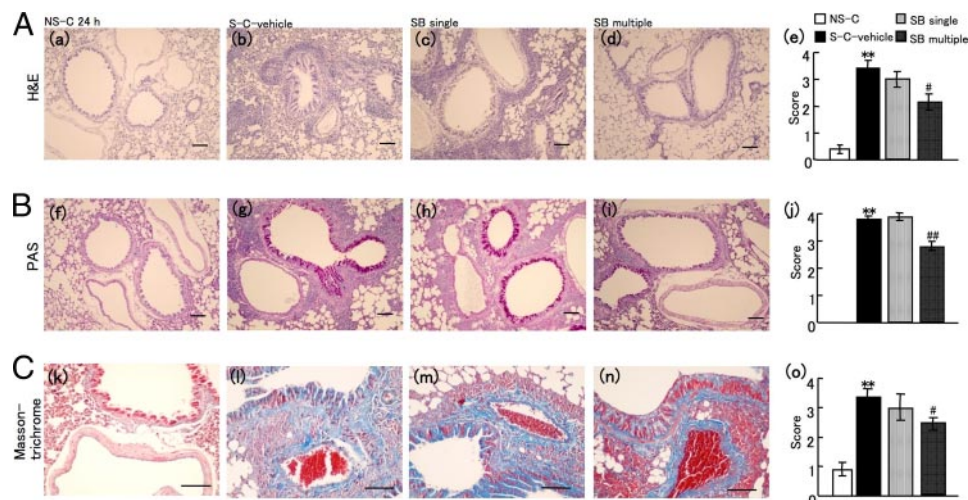


FIGURE 6. Effects of SB290157 (C3a receptor antagonist) on inflammation (H&E), goblet cell hyperplasia (PAS), and subepithelial fibrosis (Masson's trichrome) in lungs of the sensitized mice. H&E (A), PAS (B), and Masson's trichrome (C) staining 24 h after the fourth Ag challenge in nonsensitized (a, f, and k, NS-C 24 h) and sensitized (b, g, and l, S-C-vehicle) mice are shown. Additionally, the effects of treatment with a single dose (c, h, and m, SB single) or multiple doses (d, i, and n, SB multiple) of SB290157 on inflammation, goblet cell hyperplasia, and subepithelial fibrosis after the Ag challenges in sensitized mice were also evaluated. Bar shows 100 μ m. Histological appearance 24 h after the fourth challenge in the NS-C, S-C-vehicle, SB single, and SB multiple groups was scored for inflammation (e), goblet cell hyperplasia (i), and subepithelial fibrosis (o). Each value represents the mean $\pm$ SEM for five to eight animals. *, $p < 0.05$ and **, $p < 0.01$ compared with the NS-C group. #, $p < 0.05$ and ##, $p < 0.01$ compared with the S-C group.

effector phase. Neither single nor multiple treatments inhibited the production of Th2-type cytokines, although the antagonist inhibited the asthmatic features. These results show that the suppression of asthmatic features by the C3a receptor antagonist was not related to the production of Th2-type cytokines in the effector phase. However, the contribution of C3a to the production of Th2-type cytokines in experimental allergic asthma is still controversial. C3a receptor-deficient mice that were sensitized using mixed OVA and *Aspergillus fumigatus* (20) or house dust mites (21) reduced the production of Th2 cytokines as well as inhibited airway hyperresponsiveness. Indeed, it has been reported that *A. fumigatus* and house dust mites are strong activators of complement (60, 61). In contrast, in a model system using OVA adsorbed to alum, levels of Th2 cytokine production in C3a receptor-deficient and wild-type mice did not differ from each other, although the airway hyperresponsiveness was reduced in the C3a receptor-deficient mice. Taken together, it is suggested that the lack of an inhibitory effect of the C3a receptor antagonist on the production of Th2 cytokines in our model could be explained in terms of the sensitization system (OVA plus alum).

Airway remodeling, including goblet cell hyperplasia and subepithelial fibrosis, are characteristic features of severe and/or chronic asthma (9–13). These histological changes in the lung were observed in the present murine model. The development of goblet cell hyperplasia and subepithelial fibrosis was prevented by multiple treatments with the C3a receptor antagonist but not a single treatment, suggesting C3a produced in response to the first to fourth challenges to be involved in the development of airway remodeling. Consistent with the present findings, the administration of C3a induced goblet cell hyperplasia in the lung (62). Furthermore, it was reported that chronic production of IL-1 β in transgenic mice caused goblet hyperplasia and fibrosis in the lung (50). Therefore, the production of IL-1 β triggered by C3a may be an important mechanism underlying airway remodeling.

In conclusion, the present study demonstrated that: (1) multiple Ag challenges increased expression of the C3a receptor during the development of a late asthmatic response; (2) a C3a receptor antagonist suppressed the Ag-induced late asthmatic response and

airway hyperresponsiveness; (3) the mechanism leading to the development of these symptoms may correlate with the recruitment of neutrophils and/or the production of IL-1 β induced by C3a; and (4) C3a participates in the airway remodeling characterized by goblet cell hyperplasia and subepithelial fibrosis. These findings identify C3a as a potential therapeutic target for allergic asthma.

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Disclosures

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