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Immunology Letters

journal homepage: www.elsevier.com/locate/immlt



Establishment and characterization of a murine model for allergic asthma using allergen-specific IgE monoclonal antibody to study pathological roles of IgE

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ARTICLE INFO

Article history:

Received 23 May 2011

Received in revised form 5 October 2011

Accepted 16 October 2011

Available online xxx

Keywords:

IgE

Monoclonal antibody

Early asthmatic response

Late asthmatic response

Airway hyperresponsiveness

Airway remodeling

ABSTRACT

Allergen-specific IgE has long been regarded as a major molecular component of allergic asthma. Although IgE plays a central role in the early asthmatic response, its roles in the chronic phase, such as the late asthmatic response, airway hyperresponsiveness (AHR), and airway remodeling (goblet cell hyperplasia and subepithelial fibrosis) have not yet been defined well. In this study, we investigated the hypothesis that chronic responses could be induced by IgE-dependent mechanisms. BALB/c mice passively sensitized with an ovalbumin (OVA)-specific IgE monoclonal antibody (mAb) were repeatedly challenged with intratracheal administration of OVA. The first challenge induced early phase airway narrowing without any late response, but the fourth challenge caused not only an early but also a late phase response, AHR, and goblet cell hyperplasia. Macrophages, lymphocytes and neutrophils, but not eosinophils, were significantly increased in the lung 24 h after the fourth challenge. Interestingly, levels of OVA-specific IgG1 in serum increased by multiple antigen challenges. A C3a receptor antagonist inhibited the late asthmatic response, AHR, and infiltration by neutrophils. In contrast, no late response, goblet cell hyperplasia, inflammatory cells, or production of IgG1 was observed in severe combined immunodeficient mice. On the other hand, seven challenges in BALB/c mice induced subepithelial fibrosis associated with infiltration by eosinophils. In conclusion, the allergic asthmatic responses induced by passive sensitization with IgE mAb can provide a useful model system to study the pathological roles of IgE in acute and chronic phases of allergic asthma.

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1. Introduction

Patients with allergic asthma have elevated levels of antigen-specific IgE in serum, and the disease is characterized by antigen-induced early and late asthmatic responses, airway hyperresponsiveness (AHR), and airway inflammation [1–5]. IgE plays a central role in the early asthmatic response, sensitizing mast cells and/or basophils by binding to their high-affinity IgE receptors (FcεRI) [6–8] and triggering the secretion of chemical mediators within minutes after exposure to inhaled antigen [9]. The late asthmatic response, in contrast, begins several hours after exposure, and airway narrowing is associated with the migration of neutrophils, eosinophils, and lymphocytes from the bloodstream into the lung parenchyma and airway epithelium [9–11]. The antigen-induced late response is largely resolved at 24 h, at which time

there is usually AHR, measured by a reduction in the challenge concentration of methacholine (MCh), causing a 20% fall in FEV1 [4]. Furthermore, airway remodeling, including goblet cell hyperplasia and subepithelial fibrosis, is a characteristic feature, especially of chronic asthma [12–16].

Although it is generally accepted that IgE plays a primary role in triggering the early asthmatic response, it remains unclear whether the late response and airway remodeling are induced by an IgE-dependent mechanism. It has been reported that an IgE-mediated reaction was followed by inflammation of the lung leading to AHR [17,18]; however, the mechanism responsible for IgE-induced AHR remains unexplained.

In previous studies, we have reported a murine asthma model that shows early and late asthmatic responses, AHR, and airway remodeling after repeated intratracheal antigen challenges in BALB/c mice systemically sensitized with an intraperitoneal injection of ovalbumin (OVA) + alum [19,20]. In this OVA + alum model, histological examination revealed increased expression of C3a receptors in the lung following multiple antigen challenges, and the C3 level in serum was reduced during the late asthmatic response [20]. Additionally, a C3a receptor antagonist suppressed the antigen-induced late asthmatic response and AHR,

Abbreviations: sRaw, specific airway resistance; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; MCh, methacholine; PAS, periodic acid-Schiff.

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suggesting that C3a is involved in the development of the late asthmatic response and AHR [20]. However, it is difficult to understand how IgE is associated with the development of acute and chronic reactions using this OVA + alum model because not only OVA-specific IgE but also IgG1 in serum increased in response to OVA + alum even prior to the first challenge [19]. On the other hand, we have made an OVA-specific IgE monoclonal antibody (mAb) (OE-1) by establishing a B cell hybridoma producing murine IgE. The mast cell line RBL2H3 sensitized with OE-1 induced the release of β -hexosaminidase, as an index of degranulation, following activation by OVA challenge in vitro [21].

In the present study, we investigated the hypothesis that the development of allergic airway inflammation, including early and late asthmatic responses, AHR, and airway remodeling, could be induced in BALB/c mice passively sensitized with intraperitoneal injection of OE-1, and compared the responses with those in mice actively sensitized with OVA + alum. Moreover, this model was characterized by pathological analyses using a complement C3a receptor antagonist and mice carrying the severe combined immunodeficiency (SCID) mutation.

2. Materials and methods

2.1. Animals

Male 7-wk-old BALB/c and SCID (C.B-17/scid/scid) mice were obtained from Japan SLC and Japan CLEA, respectively. These mice were maintained in a temperature-controlled environment with free access to standard rodent chow and water. All of the experimental procedures were approved by the Experimental Animal Research Committee at Kobe Pharmaceutical University.

2.2. OVA-specific IgE mAb

OVA-specific IgE mAb (OE-1) was derived from a B cell hybridoma producing murine IgE as described previously [21]. The hybridoma was grown in the CELLline CL1000 with BD-Cell-MAb medium (BD Biosciences) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin–streptomycin. OE-1 levels in culture supernatants of hybridoma were assayed by ELISA. OE-1 was detected by using plates coated with anti-mouse IgE antibody and adding biotin-labeled anti-mouse IgE antibody. Alkaline phosphate anti-biotin was added, and then the plate was developed with p-nitrophenyl phosphate, and measurements were made at 405 nm using a microplate reader. OE-1 levels were calculated by comparison with mouse IgE standards (Southern Biotech).

2.3. Passive sensitization with a specific IgE mAb

As shown in Fig. 1A, BALB/c or SCID mice were passively sensitized with repeated intraperitoneal injections of a hybridoma supernatant containing OE-1 (4–100 μ g/mouse) on days 0, 1, 2, and 7. Nonsensitized mice were injected with a culture supernatant of the parental myeloma cell line. Both the sensitized and nonsensitized mice were challenged on days 1, 2, 3, and 8 under anesthesia with escaïn (Mylan Pharmaceuticals) with 1% OVA (grade V; Sigma–Aldrich) in a volume of 20 μ L by intratracheal administration as reported [19,20]. Additionally, mice sensitized with OE-1 on days 0, 1, 2, and 7 were challenged on days 1, 2, and 3 with 1% OVA, and then with saline (OE-1-sensitized-nonchallenged group) or 1% keyhole limpet hemocyanin (KLH; Sigma–Aldrich) (OE-1-sensitized-KLH-challenged group) on day 8.

Furthermore, to test whether eosinophilia and subepithelial fibrosis in the lung developed by more repeated antigen challenges, BALB/c mice were passively sensitized with an intraperitoneal

injection of OE-1 (100 μ g/mouse) on days 0, 1, 2, 7, 8, 9, and 14, and the sensitized mice were challenged on days 1, 2, 3, 8, 9, 10, and 15 with 1% OVA or 1% KLH (Fig. 8A). Furthermore, nonsensitized mice were challenged on days 1, 2, 3, 8, 9, 10, and 15 with 1% OVA.

2.4. Active sensitization with OVA + alum

Active sensitization with OVA + alum was performed according to a previously described method [20]. BALB/c mice were sensitized by intraperitoneal injection with OVA adsorbed to alum (Wako). OVA was used at a dose of 50 μ g adsorbed to 1 mg alum/0.2 mL/mouse on days 0 and 14. The sensitized mice were challenged on days 28, 29, 30, and 35 with 1% OVA in a volume of 20 μ L by intratracheal administration.

2.5. Treatment with SB290157

A C3a receptor antagonist, SB290157 (30 mg/kg) (Calbiochem), was intraperitoneally injected once 30 min before the fourth challenge. The dose of inhibitor has been reported to cause almost maximal inhibition of the induction of the late asthmatic response in actively sensitized mice [20].

2.6. Adoptive transfer of spleen cells

The spleen was isolated from naïve BALB/c mice and kept in HBSS containing 2% FBS. Single cell suspensions were prepared by pressing the spleen through a mesh screen, and red blood cells were removed by incubating spleen cells in ACK lysing buffer. Cells were washed once in HBSS containing 2% FBS, and determined by staining with Trypan blue. Cell suspensions containing 2×10^7 cells were adoptively transferred intravenously to SCID mice 1 h before the first sensitization with OE-1.

2.7. Measurement of airway resistance and AHR to MCh

Airway resistance and AHR to MCh were determined in vivo as described previously [20]. To evaluate the degree of airway resistance, specific airway resistance (sRaw; $\text{cmH}_2\text{O} \times \text{mL}/(\text{mL/s})$) 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; M.I.P.S.) according to the method of Pennock et al. [22].

AHR to MCh was assessed 24 h after the fourth antigen challenge. Briefly, increasing doses of MCh (3.125, 6.25, and 12.5 μ g/mL) in solution were consecutively applied by the intratracheal administration technique of nonsensitized and sensitized mice under escaïn anesthesia at 30-min intervals. sRaw was measured 2 min after the respective instillations of the three doses of MCh.

2.8. Analysis of cells recovered by bronchoalveolar lavage (BAL)

To evaluate airway cellular inflammation, we examined the accumulation of inflammatory cells in BAL fluid (BALF) as detailed previously [20]. Animals were killed with diethyl ether. The trachea was cannulated, and the left bronchi were tied for histological examination. The right air lumen was washed twice with 0.5 mL 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).

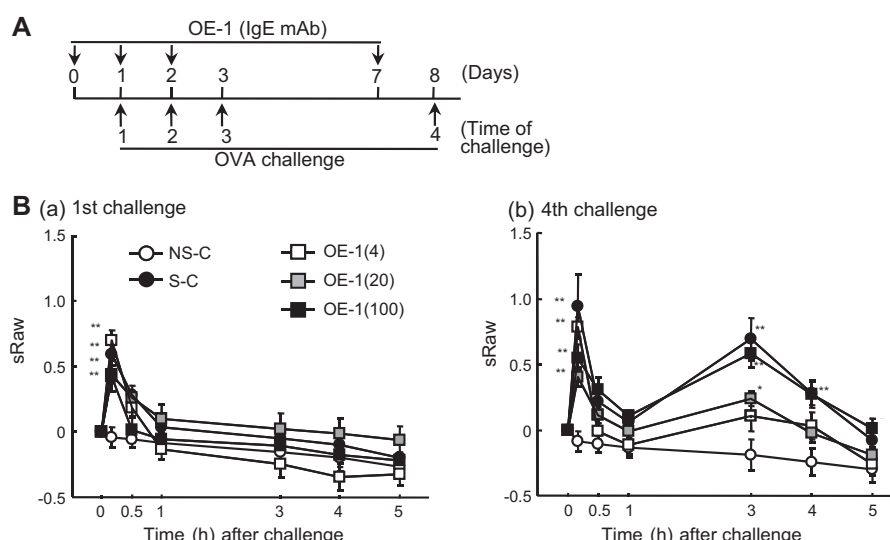


Fig. 1. Multiple antigen challenges induce a late asthmatic response in BALB/c mice passively sensitized with antigen-specific IgE mAb. (A) Experimental protocol for the sensitization with OE-1 and challenge with antigen in mice. (B) Changes in sRaw after the first (a) and fourth (b) challenges in nonsensitized-challenged (NS-C), OVA + alum-sensitized-challenged (S-C), and OE-1 (4–100 μ g/mouse)-sensitized-challenged (OE-1(4), OE-1(20), and OE-1(100)) mice. Results shown are from one experiment representative of two independent trials. Each value represents the mean $\pm$ SEM of 4–6 animals. * p < 0.05 and ** p < 0.01 compared with the NS-C group. sRaw, specific airway resistance.

2.9. 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 hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Masson's trichrome, and immunohistochemistry (C3a receptor) as described previously [20].

Immunohistochemistry (C3a receptor) was performed as follows: 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 antibody against the C3a receptor (SC-14624, 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 performed using Mayer's hematoxylin. As a negative control, goat IgG was used.

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

2.10. Measurement of OVA-specific IgE, IgG1, and IgG2a in serum

Levels of OVA-specific IgE, IgG1 and IgG2a antibodies in serum were measured by ELISA, as described [23,24]. OVA-specific IgE antibody in serum was detected using plates coated with anti-mouse IgE antibody and adding biotin-labeled OVA. Streptavidin–HRP was added, the plate was developed with TMB, and measurements were made at 450 nm using a microplate reader after stopping the reaction with sulfuric acid. Values for serum OVA-specific IgE (1:5) were expressed as absorbance units. As negative controls, mice sensitized with OE-1 were challenged with four (OE-1 (4th) without OVA) or seven saline challenges (OE-1 (7th) without OVA).

OVA-specific IgG1 or IgG2a was detected by using plates coated with OVA and adding alkaline phosphatase-conjugated anti-mouse IgG1 or IgG2a. The plates were developed with p-nitrophenyl phosphate and read at 405 nm using a microplate reader. Values for

serum OVA-specific IgG1 (1:1000) and IgG2a (1:20) were expressed as absorbance units.

2.11. Measurement of cytokines in BALF, and mouse mast cell protease-1 (mMCP-1) and complement C3 in serum

IL-4, IL-5, and IL-13 in BALF were measured using quantitative colorimetric sandwich ELISA kits (R&D Systems). The minimum detectable dose was 2, 7, and 1.5 pg/mL, respectively.

ELISA kits for mMCP-1 and C3 in serum were obtained from eBioscience and Kamiya Biomedical, respectively.

2.12. Activation of C3 in vitro by immune complexes of OVA and OVA-specific IgG1 mAb

The activation of C3 induced in vitro by immune complexes of OVA + OVA-specific IgE mAb (OE-1), OVA + OVA-specific IgG1 mAb (O1–10), and OVA + OVA-specific polyclonal Ab (pAb) was examined by ELISA with modification of the system developed by Banda et al. [25]. Diluted concentrations (1–1000 μ g/mL) of OE-1, O1–10, and pAb were detected by using plates coated with OVA and adding complement (Rockland Immunochemicals). Horseradish peroxidase-conjugated goat IgG anti-mouse C3 antibody (MP Biomedicals) was added and the color reaction was examined by adding TMB substrate at 450 nm using a microplate reader. The activation of C3 by immune complexes of OVA + OE-1, OVA + O1–10, and OVA + anti-OVA pAb was expressed as absorbance units.

The pAb was obtained as described previously [26]. The emulsion (100 μ g OVA/100 μ g complete Freund's adjuvant/0.2 mL/animal) was injected intraperitoneally into LEW/sera rats (day 0) and boosted on day 14, followed by collecting serum on day 21. OVA-specific pAb was isolated from rat serum by precipitation, dialysis, and then passing through a HiTrap affinity column (Pharmacia Biotech). OE-1 and O1–10 in cultured supernatants of hybridomas were also purified with the HiTrap affinity column.

2.13. Statistical analyses

Differences between the two groups were analyzed using Student's *t*-test. To compare more than two groups, Dunnett's test was

Table 1

Changes in sRaw after KLH challenge in BALB/c mice passively sensitized with OVA-specific IgE mAb.

	sRaw	
	Early phase (10 min)	Late phase (3 h)
NS-OVA	-0.016 ± 0.022	-0.129 ± 0.064
OE-1-NC	-0.010 ± 0.022	-0.120 ± 0.017
OE-1-KLH	-0.019 ± 0.046	-0.099 ± 0.023
OE-1-OVA	$0.810 \pm 0.231^{*,\backslash,\S}$	$0.533 \pm 0.050^{*,\backslash,\S\S}$

Mice sensitized with OE-1 (100 µg/mouse) on days 0, 1, 2, and 7 were challenged on days 1, 2, and 3 with 1% OVA, and then with saline (OE-1-NC group), 1% KLH (OE-1-KLH group), and 1% OVA (OE-1-OVA group) on day 8. Furthermore, nonsensitized mice were challenged on days 1, 2, 3, and 7 with 1% OVA (NS-OVA). sRaw was measured 10 min (early phase) and 3 h (late phase) after the fourth challenge in the groups. Results are from one experiment representative of two independent trials. Each value is the mean $\pm$ SEM of 4–6 animals. sRaw, specific airway resistance; KLH, keyhole limpet hemocyanin.

* $p < 0.05$ compared with the NS-OVA group.

** $p < 0.01$ compared with the NS-OVA group.

\ $p < 0.05$ compared with the OE-1-NC group.

\ \ $p < 0.01$ compared with the OE-1-NC group.

\ $p < 0.05$ compared with the OE-1-KLH group.

\ \ $p < 0.01$ compared with the OE-1-KLH group.

used after conducting one-way analysis of variance (ANOVA). A probability value of $p < 0.05$ was considered significant.

3. Results

3.1. Multiple antigen challenges induce a late asthmatic response in mice passively sensitized with antigen-specific IgE mAb

First, we investigated whether a late asthmatic response is induced even in mice passively sensitized with an OVA-specific IgE mAb (OE-1) after challenges with OVA, and compared the results with those in mice actively sensitized with OVA + alum. Fig. 1B shows the time course of changes in specific airway resistance (sRaw) after the first and fourth challenges in passively sensitized BALB/c mice. The first challenge caused a temporary increase in sRaw, peaking at 10 min, but no late response was induced in mice sensitized with OE-1 at any doses used (Fig. 1Ba). In contrast, the fourth challenge induced a biphasic increase in sRaw, peaking at 10 min and 3 h (Fig. 1Bb), and a significant late phase response was observed in mice sensitized with OE-1 at 20 and 100 µg/mouse. The extent of the late asthmatic response in mice given 100 µg OE-1 was comparable to that in mice actively sensitized with OVA + alum. Furthermore, in nonsensitized-challenged, OE-1 (100 µg/mouse)-sensitized-nonchallenged, and OE-1 (100 µg/mouse)-sensitized-KLH-challenged groups, no obvious changes in sRaw were observed at 10 min and 3 h (Table 1).

sRaw values before the fourth challenge were 2.303 ± 0.061 in the nonsensitized-challenged, 2.262 ± 0.082 , 2.271 ± 0.081 , and 2.264 ± 0.054 in OE-1 (4, 20, and 100 µg/mouse)-sensitized-challenged, and 2.371 ± 0.104 in OVA + alum-sensitized-challenged groups. Throughout the above experiment, no significant differences in sRaw before the challenges were found among the groups.

3.2. Multiple antigen challenges induce infiltration of inflammatory cells into the lung of mice passively sensitized with antigen-specific IgE mAb

To investigate airway inflammation, BALF cells were collected 24 h after the fourth challenge in mice passively sensitized with OE-1. Numbers of inflammatory cells, such as macrophages, lymphocytes, and neutrophils were significantly increased in BALF after the fourth challenge in mice sensitized with OE-1 at 100 µg/mouse; however, the recruitment of eosinophils was only slightly induced

Table 2

Changes in neutrophil numbers in BALF after KLH challenge in BALB/c mice passively sensitized with OVA-specific IgE mAb.

	Number of cells ($\times 10^4$ cells/BALF)	
	Total cells	Neutrophils
NS-OVA	1.58 ± 0.25	0.10 ± 0.04
OE-1-NC	$4.16 \pm 0.26^{**}$	0.15 ± 0.03
OE-1-KLH	$3.94 \pm 0.19^{**}$	0.14 ± 0.03
OE-1-OVA	$4.26 \pm 0.20^{**}$	$1.06 \pm 0.13^{*,\backslash,\S\S}$

Mice sensitized with OE-1 (100 µg/mouse) on days 0, 1, 2, and 7 were challenged on days 1, 2, and 3 with 1% OVA, and then with saline (OE-1-NC group), 1% KLH (OE-1-KLH group), and 1% OVA (OE-1-OVA group) on day 8. Furthermore, nonsensitized mice were challenged on days 1, 2, 3, and 7 with 1% OVA (NS-OVA). BALF cells were collected 24 h after the fourth challenge in the groups. Results are from one experiment representative of two independent trials. Each value is the mean $\pm$ SEM of 4–6 animals. KLH, keyhole limpet hemocyanin.

** $p < 0.01$ compared with the NS-OVA group.

\ \ $p < 0.01$ compared with the OE-1-NC group.

\ \ $p < 0.01$ compared with the OE-1-KLH group.

after the fourth challenge in passively sensitized mice, even with 100 µg/mouse of OE-1, although marked eosinophilia was induced in actively sensitized mice (Fig. 2A). When mice were sensitized with OE-1 at 4 and 20 µg/mouse, no marked increase in inflammatory cells in BALF was observed even after the fourth challenge although a slight increase in neutrophils was induced in mice sensitized with OE-1 at 20 µg/mouse. Fig. 2B shows time-course changes in the numbers of macrophages, lymphocytes, neutrophils, and eosinophils in BALF after the fourth challenge in mice sensitized with OE-1 (100 µg/mouse). Macrophages and lymphocytes had infiltrated the lung before the fourth challenge, and did not increase further 4 and 24 h after the challenge. Although the number of neutrophils was small prior to the fourth challenge, it was significantly elevated 4 and 24 h after the challenge. Furthermore, the infiltration of neutrophils in BALF 24 h after the fourth challenge with KLH instead of OVA in mice sensitized with OE-1 was not observed (Table 2).

3.3. Multiple antigen challenges induce production of IL-4 and IL-13 in BALF of mice passively sensitized with antigen-specific IgE mAb

To investigate the role of Th2-type cytokines, we measured the levels of IL-4, IL-5, and IL-13 in BALF of mice sensitized with OE-1. The levels of IL-4 and IL-13 in BALF 24 h after the fourth antigen challenge in mice sensitized with OE-1 were significantly increased in comparison with those in OE-1-sensitized-nonchallenged mice, although these cytokines were not detected in nonsensitized-challenged mice (Table 3). In addition, no significant differences in the levels of IL-4 and IL-13 were found

Table 3

Changes in levels of IL-4 and IL-13 in BALF after KLH challenge in BALB/c mice passively sensitized with OVA-specific IgE mAb.

	IL-4	IL-13
NS-OVA	ND	ND
OE-1-NC	3.0 ± 0.4	5.0 ± 1.2
OE-1-KLH	3.2 ± 0.3	4.5 ± 0.8
OE-1-OVA	$13.7 \pm 4.2^{\backslash,\S}$	$19.8 \pm 5.8^{\backslash,\S}$

Mice sensitized with OE-1 (100 µg/mouse) on days 0, 1, 2, and 7 were challenged on days 1, 2, and 3 with 1% OVA, and then with saline (OE-1-NC group), 1% KLH (OE-1-KLH group), and 1% OVA (OE-1-OVA group) on day 8. Furthermore, nonsensitized mice were challenged on days 1, 2, 3, and 7 with 1% OVA (NS-OVA). BALF were collected 24 h after the fourth challenge in the groups. Results are from one experiment representative of two independent trials. Each value is the mean $\pm$ SEM of 4–6 animals. KLH, keyhole limpet hemocyanin; ND, not detectable.

\ $p < 0.05$ compared with the OE-1-NC group.

\ $p < 0.05$ compared with the OE-1-KLH group.

Table 4

Changes in mMCP-1 in serum of BALB/c mice passively sensitized with antigen-specific IgE mAb.

	sRaw	mMCP-1 (ng/mL)
NS-C	-0.019 ± 0.024	6.6 ± 1.7
OE-1-NC	0.003 ± 0.025	5.7 ± 0.6
OE-1-C	$0.996 \pm 0.253^{**,\backslash\backslash}$	$17.2 \pm 2.5^{*,\backslash\backslash}$

sRaw was measured 10 min after the first challenge in OE-1 (100 μ g/mouse)-sensitized-challenged (OE-1-C) mice. Negative controls were nonsensitized-challenged (NS-C) and OE-1-sensitized-nonchallenged (OE-1-NC) mice. Furthermore, the level of mMCP-1 in serum was measured 30 min after the challenge. Results are from one experiment representative of two independent trials. Each value is the mean $\pm$ SEM of 5 animals. sRaw, specific airway resistance; mMCP-1, mouse mast cell protease-1.

* $p < 0.05$ compared with the NS-C group.

** $p < 0.01$ compared with the NS-C group.

\backslash\backslash $p < 0.01$ compared with the OE-1-NC group.

between OE-1-sensitized-nonchallenged and OE-1-sensitized-KLH challenged groups (Table 3). The level of IL-5 in BALF 24 h after the fourth antigen challenge in mice sensitized with OE-1 was not detectable (data not shown).

3.4. Multiple antigen challenges induce production of OVA-specific IgG1 in mice passively sensitized with antigen-specific IgE mAb

It has been reported that IgE mAb passively administered to mice together with the antigen can upregulate the production of antigen-specific IgG [27–34]; therefore, we measured IgG1 and IgG2a in the serum of mice passively sensitized with OE-1. The level of OVA-specific IgG1, but not IgG2a, in serum was significantly increased 24 h after the fourth challenge (day 9) in mice sensitized with OE-1 at 20 and 100 μ g/mouse (Fig. 3Aa and b). Increases in IgG1 production in mice sensitized with OE-1 (100 μ g/mouse) was observed on days 6 and 9 (Fig. 3B).

3.5. Change in the levels of mMCP-1 in serum

In order to examine the participation of mast cells in the early asthmatic response, we measured the serum level of mMCP-1, a marker of mast cell activation, 30 min after the first antigen challenge in mice sensitized with OE-1. The increase in sRaw was observed at 10 min after the first antigen challenge, and the level of mMCP-1 in serum at 30 min was greater than

in nonsensitized-challenged and OE-1-sensitized-nonchallenged groups (Table 4).

3.6. Changes in the expression of C3a receptors in the lung and complement C3 level in serum

We have reported that the level of C3a receptor expression was increased in the lung following multiple OVA challenges, and the C3 level in serum was reduced during the late asthmatic response in mice actively sensitized with OVA + alum [20]. Accordingly, we investigated whether the complement system also works in the IgE-sensitized model. As expected, C3a receptors were markedly expressed in the lung 24 h after the fourth challenge in comparison with that in nonsensitized mice, and the expression was especially increased on infiltrated inflammatory cells (Fig. 4Aa and b). Additionally, the increased expression developed prior to the fourth challenge (Fig. 4Ac).

Fig. 4Ba and b show the time-course changes in C3 and OVA-specific IgG1 levels in serum after the fourth challenge in OE-1-sensitized mice, respectively. Both levels were reduced at 4 h after the fourth challenge compared with those before the fourth challenge, and then restored at 24 h.

Because both C3 and IgG1 levels were decreased during the late asthmatic response, we examined whether immune complexes of the antigen and antibodies are capable of activating the complement system in vitro. To investigate the role of OVA-specific antibodies in the activation of C3, the level of C3b was measured as an indicator of C3a production. As shown in Fig. 4C, C3b was produced from a standard complement in the presence of either an immune complex composed of OVA-specific IgG1 mAb (O1–10) and OVA, or OVA-specific pAb and OVA in a concentration-dependent manner; however, an immune complex composed of an OVA-specific IgE mAb (OE-1) and OVA did not produce C3b.

3.7. C3a receptor antagonist SB290157 inhibits a late asthmatic response, AHR, and inflammatory cells in BALF

To test whether the biphasic asthmatic response, AHR, and inflammatory cells in BALF in the IgE-sensitized model is inhibited by systemic treatment with a C3a receptor antagonist, we analyzed the effects of SB290157. The treatment inhibited the late response and AHR, but not the early response (Fig. 5A and B). Furthermore, treatment with SB290157 significantly inhibited the increase in neutrophils, but had no effect on increased numbers of macrophages and lymphocytes (Fig. 5C).

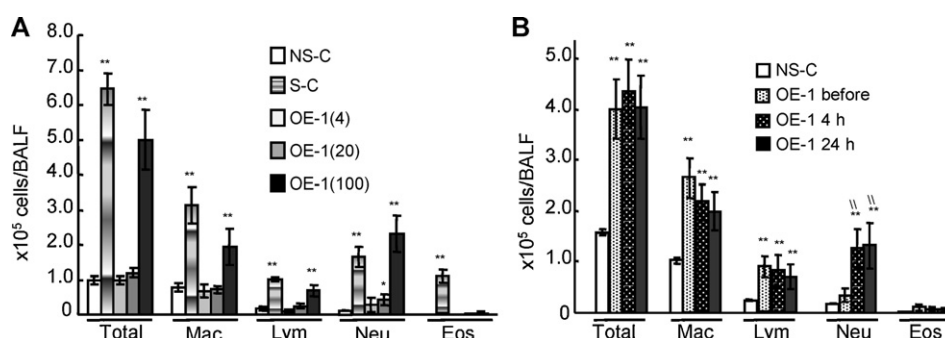


Fig. 2. Multiple antigen challenges induce infiltration of inflammatory cells into the lungs of BALB/c mice passively sensitized with antigen-specific IgE mAb. (A) Changes in inflammatory cell numbers in BALF 24 h after the fourth challenge in nonsensitized-challenged (NS-C), OVA + alum-sensitized-challenged (S-C), and OE-1 (4–100 μ g/mouse)-sensitized-challenged (OE-1(4), OE-1(20), and OE-1(100)) mice. (B) Changes in inflammatory cell numbers in BALF 24 h after the fourth challenge in NS-C and 2 h before (OE-1 before) or 4 h (OE-1 4 h) and 24 h (OE-1 24 h) after the fourth challenge in mice sensitized with OE-1 (100 μ g/mouse). Results shown are from one experiment representative of two independent trials. Each value represents the mean $\pm$ SEM of 4–6 animals. * $p < 0.05$ and ** $p < 0.01$ compared with the NS-C group. \backslash\backslash $p < 0.01$ compared with the OE-1 before group. Total, all cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

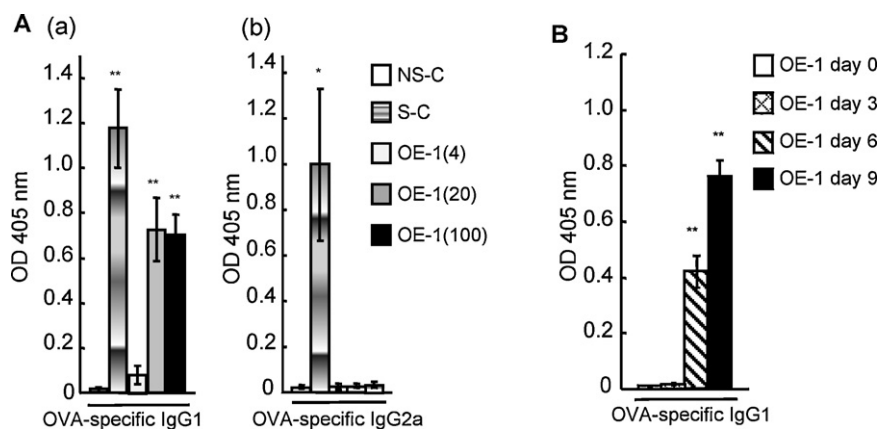


Fig. 3. Multiple antigen challenges increase antigen-specific IgG1 production in serum of BALB/c mice passively sensitized with antigen-specific IgE mAb. (A) Levels of OVA-specific IgG1 (a) and IgG2a (b) in serum 24 h after the fourth challenge in nonsensitized-challenged (NS-C), OVA+alum-sensitized-challenged (S-C), and OE-1 (4–100 μ g/mouse)-sensitized-challenged (OE-1(4), OE-1(20), and OE-1(100)) mice. (B) Time-course of changes in OVA-specific IgG1 levels in serum during antigen challenges in mice sensitized with OE-1 (100 μ g/mouse). Results shown are from one experiment representative of two independent trials. Each value represents the mean $\pm$ SEM of 4–6 animals. * p < 0.05 and ** p < 0.01 compared with the NS-C or day 0 group.

3.8. SCID mice passively sensitized with a specific IgE mAb fail to exhibit a late response, AHR and OVA-specific IgG1 production

We investigated whether the late asthmatic response in the IgE-sensitized model is associated with the interaction between B and T lymphocytes during multiple challenges with OVA. SCID mice, which lack mature and functional B and T lymphocytes, were sensitized with OE-1 and then intratracheally challenged, as were BALB/c mice. Sensitized SCID mice showed an early phase response after both the first and fourth challenges (Fig. 6Aa and b), but no late asthmatic response, recruitment of inflammatory cells in BALF, or OVA-specific IgG1 production (Fig. 6Ab, B, and C); however, when

spleen cells isolated from naïve BALB/c mice were adoptively transferred to SCID mice 1 h before the first sensitization with OE-1, a late asthmatic response, the recruitment of inflammatory cells (macrophages, lymphocytes and neutrophils) in BALF, and IgG1 production, was observed (Fig. 6Ab, B, and C).

3.9. SCID mice passively sensitized with a specific IgE mAb fail to exhibit goblet cell hyperplasia

We subsequently examined whether airway inflammation and goblet cell hyperplasia in the lungs of SCID mice sensitized with OE-1 were observed. As defined by histological analysis using

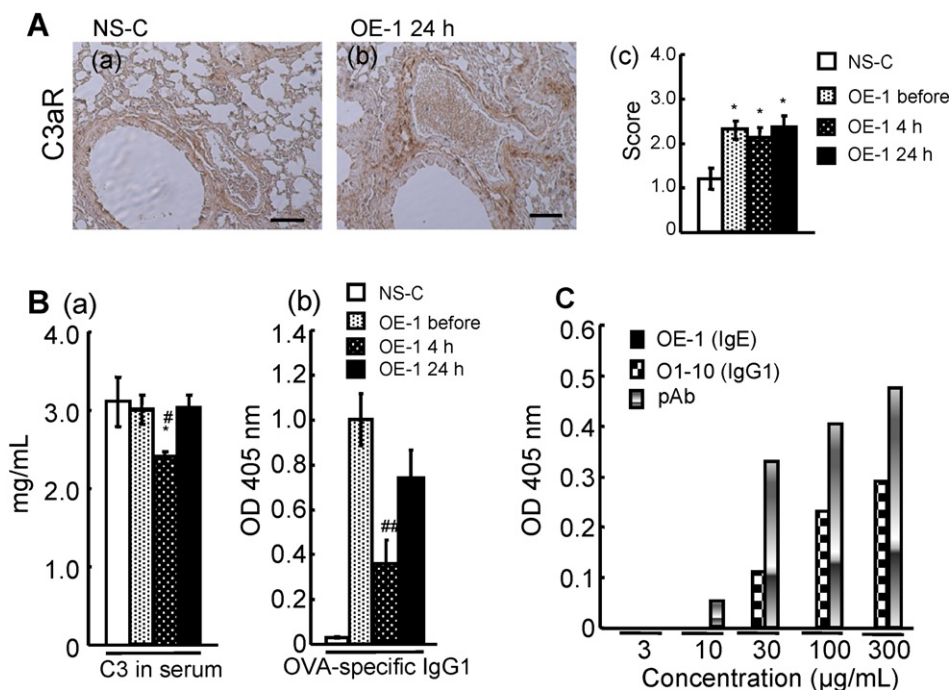


Fig. 4. Changes in the expression of C3a receptors in the lung and C3 level in serum of BALB/c mice passively sensitized with antigen-specific IgE mAb. (A) Immunohistochemical localization of C3a receptors in lung tissue 24 h after the fourth challenge in nonsensitized-challenged (a, NS-C) and OE-1-sensitized-challenged (b, OE-1 24 h) mice. Bar shows 100 μ m. Histological appearance was scored for C3a receptor expression (c). (B) C3 (a) and IgG1 (b) levels in serum 24 h after the fourth challenge in nonsensitized (NS-C) mice, and 2 h before (OE-1 before) or 4 h (OE-1 4 h) and 24 h (OE-1 24 h) after the fourth challenge in mice sensitized with OE-1. Results shown are from one experiment representative of two independent trials. Each value represents the mean $\pm$ SEM of 4–5 animals. * p < 0.05 compared with the NS-C group. # p < 0.05 and ## p < 0.01 compared with the OE-1 before group. (C) C3 activation induced in vitro by the OVA and anti-OVA IgG1 mAb (O1-10) immune complex was evaluated. Results shown are from one experiment representative of four independent trials.

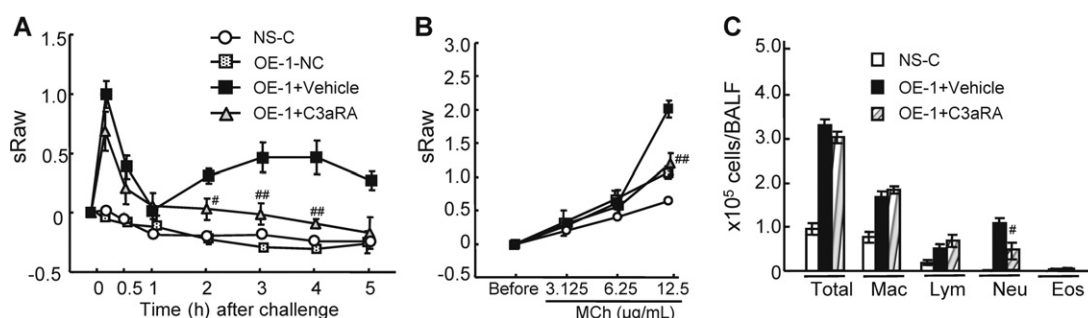


Fig. 5. Effect of SB290157 (C3a receptor antagonist) on the late asthmatic response (A), AHR (B), and inflammatory cells in BALF (C) after the fourth antigen challenge in BALB/c mice passively sensitized with antigen-specific IgE mAb. A C3a receptor antagonist, SB290157 (30 mg/kg), was intraperitoneally injected once 30 min before the fourth challenge (OE-1 + C3aRA). Negative controls were nonsensitized-challenged (NS-C) and OE-1-sensitized-nonchallenged (OE-1-NC) mice. The positive control was OE-1-sensitized-challenged, vehicle-treated (OE-1 + vehicle) mice. Results shown are from one experiment representative of two independent trials. Each value represents the mean $\pm$ SEM of 5 animals. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the OE-1-vehicle group. sRaw, specific airway resistance; AHR, airway hyperresponsiveness; Total, all cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

H&E staining, little leukocyte recruitment was observed 24 h after the fourth challenge in nonsensitized BALB/c mice (Fig. 7Aa). In contrast, there was marked infiltration of leukocytes around the blood vessels and airways 24 h after the fourth challenge in OE-1-sensitized mice (Fig. 7Ab). Additionally, we performed a histological examination with PAS staining for the detection of goblet cells (Fig. 7B). In the lungs of sensitized BALB/c mice, goblet cell hyperplasia was observed 24 h after the fourth challenge (Fig. 7Bb); however, in nonsensitized BALB/c mice, goblet cell hyperplasia was not observed (Fig. 7Ba). On the other hand, although neither lung inflammation nor goblet cell hyperplasia was observed in SCID mice sensitized with OE-1, the mice that received the spleen cells did exhibit such histological changes (Fig. 7A and B).

Histological analyses revealed that infiltration by leukocytes and goblet cell hyperplasia in the lung were markedly induced before the fourth challenge in IgE-sensitized BALB/c mice in comparison with nonsensitized-challenged mice (data not shown). These histological changes continued for at least 24 h.

3.10. Multiple antigen challenges induce infiltration by eosinophils and subepithelial fibrosis in the lungs of mice passively sensitized with antigen-specific IgE mAb

To investigate whether more than four antigen challenges induced infiltration by eosinophils and subepithelial fibrosis in the lungs of the IgE-sensitized model, we analyzed these responses at the seventh antigen challenge, and compared the results with those

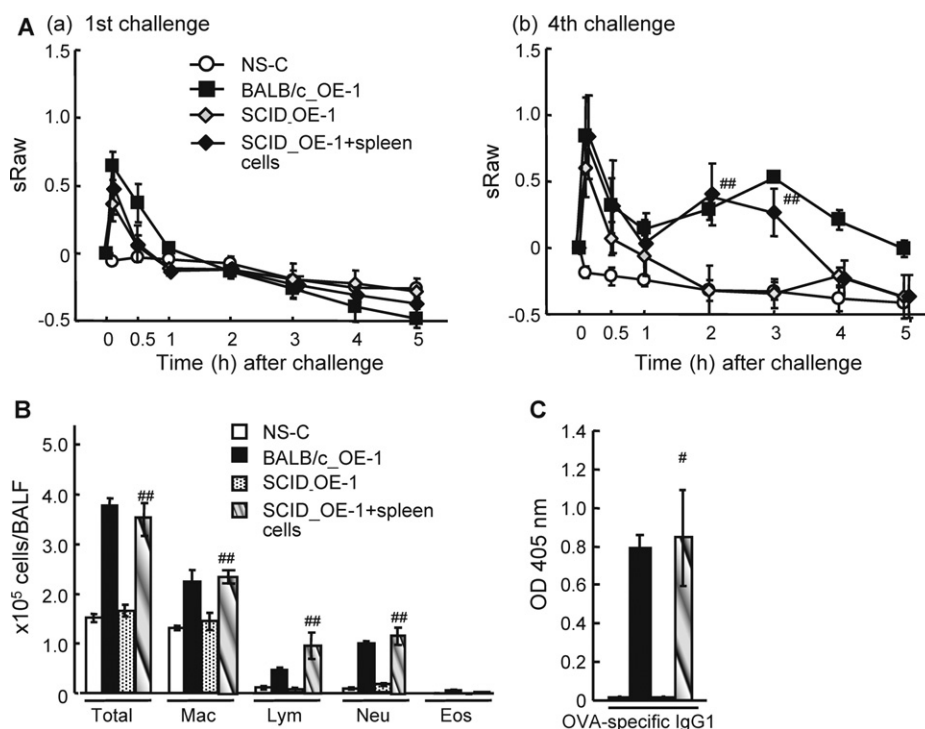


Fig. 6. SCID mice passively sensitized with antigen-specific IgE mAb fail to exhibit the antigen-induced late asthmatic response. (A) Changes in sRaw after the first (a) and fourth (b) challenges in OE-1-sensitized SCID mice, which were treated with 2% FBS-HBSS (SCID.OE-1) or spleen cells (SCID.OE-1 + spleen cells). Negative and positive controls were nonsensitized-challenged (NS-C) or OE-1-sensitized-challenged BALB/c mice (BALB/c.OE-1), respectively. (B) Changes in inflammatory cell numbers in BALF 24 h after the fourth challenge in the groups. (C) Level of OVA-specific IgG1 in serum after the fourth challenge in the groups. Results shown are from one experiment representative of two independent trials. Each value represents the mean $\pm$ SEM of 4 animals. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the SCID.OE-1 group. sRaw, specific airway resistance; Total, all cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

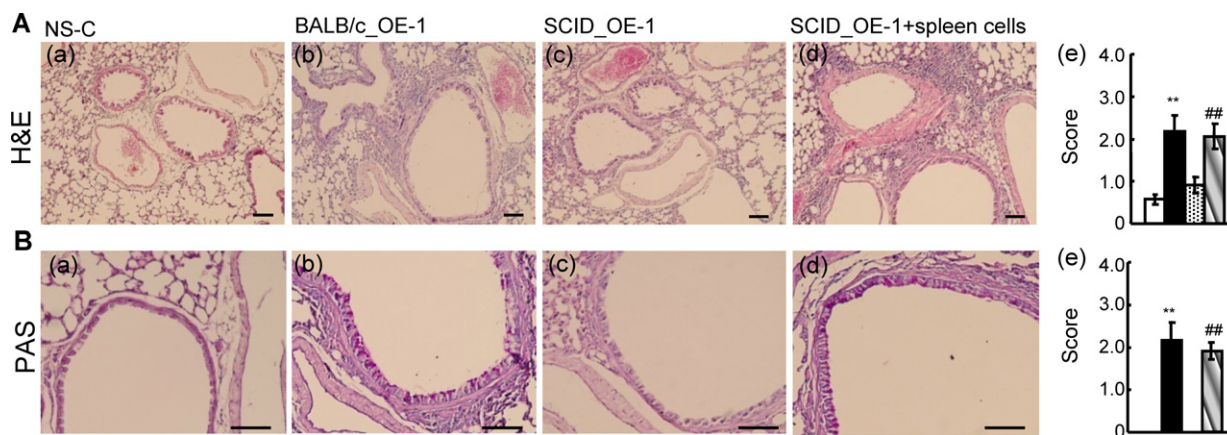


Fig. 7. SCID mice passively sensitized with the antigen-specific IgE mAb fail to exhibit antigen-induced goblet cell hyperplasia. (A) Changes in inflammation (H&E) in the lungs 24 h after the fourth challenge in OE-1-sensitized SCID mice, which were treated with 2% FBS-HBSS (c, SCID.OE-1) or spleen cells (d, SCID.OE-1 + spleen cells). Negative and positive controls were nonsensitized-challenged (a, NS-C) or OE-1-sensitized-challenged BALB/c mice (b, BALB/c.OE-1), respectively. Bar shows 100 μ m. Histological appearance was scored for inflammation (e). (B) Changes in goblet cells (PAS) in the lung 24 h after the fourth challenge in the groups. Bar shows 100 μ m. Histological appearance was scored for goblet cell hyperplasia (e). Results shown are from one experiment representative of two independent trials. Each value represents the mean $\pm$ SEM of 4 animals. ** p < 0.01 compared with the NS-C group. # p < 0.05 and ## p < 0.01 compared with the SCID.OE-1 group.

at the fourth challenge in mice actively sensitized with OVA + alum. The seventh challenge caused the infiltration of eosinophils into the lung (Fig. 8B) and subepithelial fibrosis (Fig. 8F), although these pathological changes were not significantly observed after the fourth challenge. At the seventh challenge, the production of OVA-specific IgG1 and the development of goblet cell hyperplasia were greater than those at the fourth challenge (Fig. 8C and D). In addition, the levels of OVA-specific IgE at the fourth and seventh challenge were significantly increased over the injection levels of OE-1 alone, although there was no significant difference between the two groups. The seventh antigen challenge also induced a biphasic asthmatic response (data not shown).

To investigate the role of Th2-type cytokines in this IgE-sensitized model, we measured the levels of IL-4, IL-5, and IL-13 in BALF 24 h after the fourth and seventh challenge in mice sensitized with OE-1. The levels of IL-4 and IL-13 were observed at the fourth challenge, and at the seventh challenge were greater than at the fourth challenge (Fig. 8Da and c). Moreover, IL-5 production was observed at the seventh challenge, but not at the fourth challenge (Fig. 8Db).

Throughout the above experiments, the extents of these responses, such as eosinophil infiltration, OVA-specific IgG1 and IgE production, Th2 cytokine production, and airway remodeling (goblet cell hyperplasia and subepithelial fibrosis), at the seventh antigen challenge in mice sensitized with OE-1 were mostly similar to those at the fourth challenge in mice actively sensitized with OVA + alum (Fig. 8). Furthermore, no significant differences in these responses were found between nonsensitized-OVA-challenged and OE-1-sensitized-KLH-challenged groups (Fig. 8).

4. Discussion

Elevated allergen-specific IgE levels in serum are the hallmark of allergic asthma. Although IgE plays a central role in the early asthmatic response, its roles in the chronic phase, such as the late asthmatic response, AHR, and airway remodeling have not yet been defined well. In the present experiments, we found that, in mice passively sensitized with OVA-specific IgE mAb, the first administration of antigen induced an early asthmatic response without a late response, but the fourth challenge caused not only an early but also a late asthmatic response, AHR, and goblet cell hyperplasia. In BALF, inflammatory cells such as macrophages, lymphocytes, and neutrophils, but not eosinophils, were observed. More

interestingly, the level of OVA-specific IgG1 in serum was increased with multiple challenges. In addition, further repeated antigen challenges significantly induced subepithelial fibrosis coinciding with the infiltration by eosinophils.

We have reported that C3a was involved in the development of a late asthmatic response and AHR in the OVA + alum model [20]. Thus, its involvement in asthmatic responses in the IgE-sensitized model was also assessed. In the in vivo model of asthma, the level of C3 in serum was reduced 4 h after the fourth administration of antigen, and returned to the baseline level by 24 h (Fig. 4Ba), suggesting that C3a was derived from the proteolytic cleavage of C3 during the late phase response. In in vitro experiments, the immune complex of OVA-specific IgG1 mAb and OVA, but not OVA-specific IgE mAb and OVA, activated C3, leading to the formation of C3a (Fig. 4C). Furthermore, the IgG1 level in serum was reduced 4 h after the fourth challenge in this IgE model (Fig. 4Bb). This decrease suggests that OVA specific-IgG1 antibody in serum may be consumed as a result of the formation of an immune complex between the IgG1 antibody and the antigen; however, since it is generally known that mouse IgG1 antibody is a poor activator of complement, the detailed mechanisms of C3 activation during the late phase should be examined in the future.

Consistent with the findings in actively sensitized murine models of asthma [20,35], C3a receptor expression was increased in epithelial cells, smooth muscle cells, and inflammatory cells in the IgE-sensitized model (Fig. 4A). In particular, the expression of C3a receptors was strongly increased in inflammatory cells in the lung. Additionally, both increases in the C3a receptor expression and inflammatory cell numbers had already occurred by the fourth challenge. Furthermore, human C3a receptors were also reported to be expressed on neutrophils, monocytes and T lymphocytes [36,37], suggesting that C3a receptor expressed on inflammatory cells was not surprising.

C3a receptor antagonist, SB290157, inhibited a late asthmatic response and AHR in both actively sensitized mice [20] and passively sensitized mice in this study (Fig. 5). Moreover, the treatment significantly inhibited the recruitment of neutrophils into the lung. In both models, the recruitment of neutrophils was consistently observed during the late asthmatic response after the fourth challenge, although only a small number of neutrophils was seen before the challenge [20,38] (Fig. 2B); therefore, the recruitment of neutrophils may trigger the late asthmatic response and AHR in these models. Indeed, we have demonstrated that an anti-Gr-1 mAb

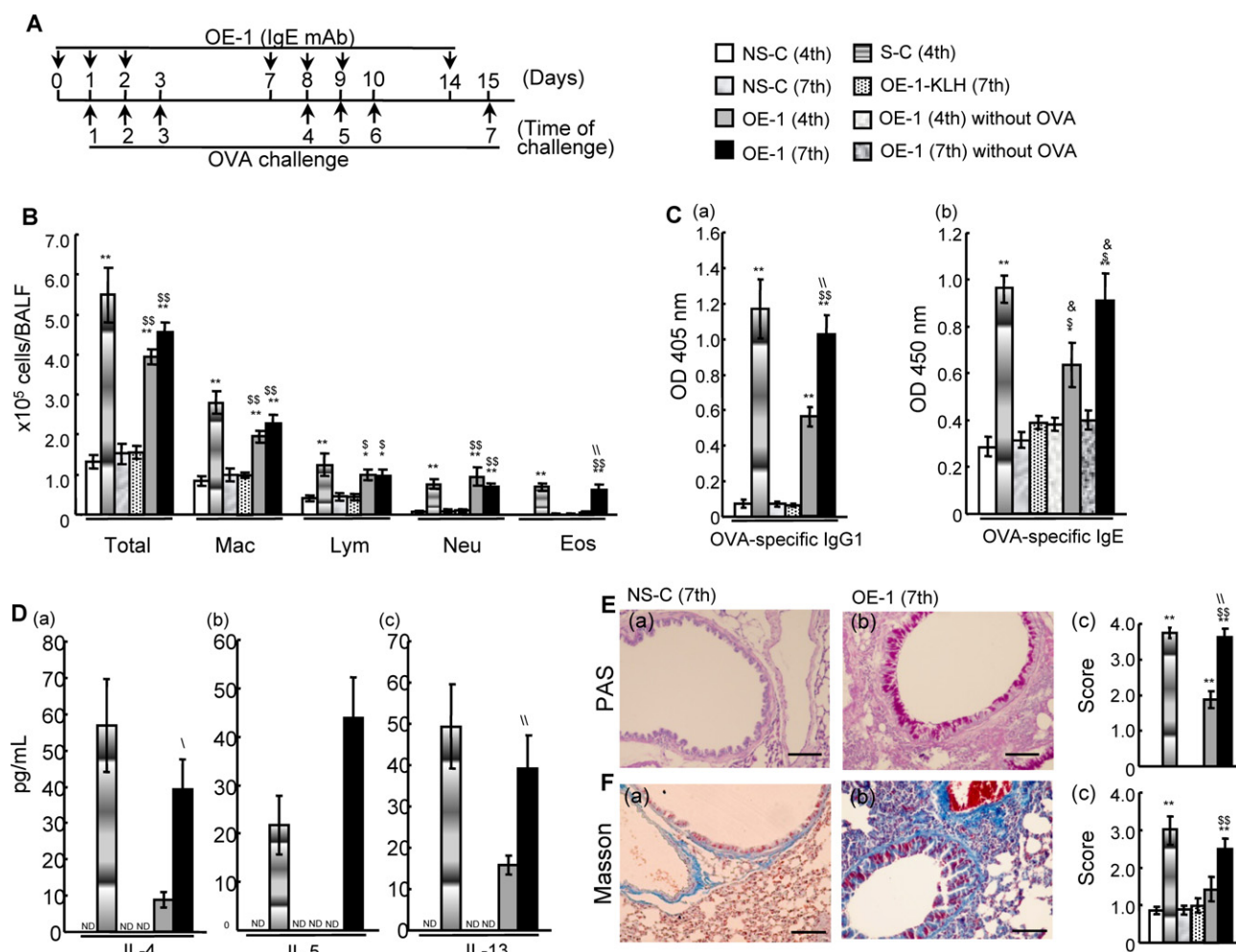


Fig. 8. Multiple antigen challenges induce subepithelial fibrosis in the lungs of BALB/c mice passively sensitized with antigen-specific IgE mAb. (A) Experimental protocol for the sensitization with OE-1 and challenge with antigen in BALB/c mice. (B) Changes in inflammatory cell numbers in BALF 24 h after the seventh OVA challenge in mice nonsensitized-challenged (NS-C (7th)), and 24 h after the seventh KLH challenge (OE-1-KLH (7th)), 24 h after the fourth (OE-1-OVA (4th)) or seventh OVA challenge (OE-1-OVA (7th)) in mice sensitized with OE-1. Additionally, the changes in nonsensitized-four OVA challenged (NS-C (4th)) and OVA + alum-sensitized-four OVA challenged (S-C (4th)) groups were evaluated. (C) Levels of OVA-specific IgG1 (a) and IgE (b) in serum 24 h after the fourth and seventh challenge in the groups. In the measurement of IgE levels, the mice sensitized with OE-1 were challenged with four (OE-1 (4th) without OVA) or seven (OE-1 (7th) without OVA) saline challenges as negative controls. (D) Levels of IL-4, IL-5, and IL-13 in BALF 24 h after the fourth and seventh challenge in the groups. (E) Changes in goblet cells (PAS) in lung tissue 24 h after the seventh challenge in the NS-C (7th) (a) and OE-1 (7th) (b) groups. Bar shows 100 μ m. Histological appearance was scored for goblet cell hyperplasia (c). (F) Changes in subepithelial fibrosis (Masson's trichrome) 24 h after the seventh challenge in the NS-C (7th) (a) and OE-1 (7th) (b) groups. Bar shows 100 μ m. Histological appearance was scored for subepithelial fibrosis (c). Results shown are from one experiment representative of two independent trials. Each value represents the mean $\pm$ SEM of 4–6 animals. * p < 0.05 and ** p < 0.01 compared with the NS-C (4th) or NS-C (7th) group. § p < 0.05 and §§ p < 0.01 compared with the OE-1-KLH (7th) group. \p < 0.05 and \p < 0.01 compared with the OE-1 (4th) group. § p < 0.05 compared with the OE-1 (4th) without OVA or OE-1 (7th) without OVA group. Total, all cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils; KLH, keyhole limpet hemocyanin; ND, not detectable.

that selectively depleted neutrophils suppressed the late asthmatic response in actively sensitized mice [38]. In contrast, an anti-IL-5 mAb that selectively suppressed eosinophilia did not exert any effect on the late response [38]. Thus, it is not surprising that the infiltration by neutrophils may have contributed to the late asthmatic response and AHR in the IgE-sensitized model as well. Actually, we found that no significant infiltration by eosinophils in BALF was induced in the IgE model at the fourth challenge even though both the late response and AHR had already developed at the fourth challenge (Figs. 1Bb, 2, and 5B). Furthermore, the fact that activated neutrophils can 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 [39,40].

Although asthma has long been considered an eosinophilic bronchitis, asthmatic symptoms in patients were not ameliorated by a marked reduction in eosinophil numbers in the circulation and

the airway tissue caused by humanized anti-IL-5 mAbs [41,42]. The recognition that some asthmatics, particularly those with severe asthma [43–45] and resistant to corticosteroids [46], have raised neutrophil counts in their airways has led to suggestions that neutrophils may be a more valid target than eosinophils. The clinical evidence supports the consideration that neutrophils can be major contributing cells to the late asthmatic response in the present murine asthma.

On the other hand, it has been reported in the murine model, in which BALB/c mice sensitized with OVA-specific IgE mAb on two consecutive days were exposed to nebulized 1% OVA for 20 min daily, 3 and 4 days after the last injection of antibody, that not only AHR but also infiltration by eosinophils was observed [18], and that the development of AHR by IgE mAb was associated with the accumulation of eosinophils by IL-5 following allergen challenge [47]. Conversely, in our IgE-sensitized model, infiltration by eosinophils and the production of IL-5 were not significantly increased at the

fourth antigen challenge, even though AHR had already developed (Figs. 2, 5B, and 8D). As suggested by Kumar and Forster [48], the roles of cells and molecules in AHR were altered by changing the protocols (dose and duration) for antigen challenge. In our IgE-sensitized model, antigen challenge was performed using the intratracheal challenge method instead of inhalation of the antigen as an aerosol mist. Thus, these different protocols may change the roles of cells and molecules in IgE mAb-induced allergic responses.

Experiments *in vivo* have shown that immune complexes composed of an antigen and IgE antibody, binding to CD23 on the surface of B cells [27–33] and/or FcεRI on the basophils [34], could be internalized more efficiently than antigens not combined with antibodies, and then antigenic peptides are presented by these cells to specific CD4⁺ T cells, which proliferate and interact with antigen-specific B cells. These findings led us to investigate whether a late asthmatic response occurs in SCID mice, which lack mature and functional T and B lymphocytes. Neither a late response, inflammatory cells, nor IgG1 production was observed in SCID mice sensitized with IgE mAb, whereas these responses occurred following the adoptive transfer of spleen cells isolated from a normal mouse (Fig. 6), suggesting that the interaction between T and B cells was required for IgE-enhanced IgG1 production, recruitment of inflammatory cells, and the development of a late asthmatic response in this model. Additionally, the increases of Th2-type cytokines, including IL-4 and IL-13, at the fourth challenge in BALF of IgE-sensitized BALB/c mice were observed (Fig. 8D). Based on these cytokine profiles, we show that the IgE-induced late asthmatic response is related to Th2-type responses.

In our experimental IgE-sensitized model, the first challenge induced only an early response, but the fourth challenge produced early and late responses (Fig. 1B). It has been reported that OE-1 induced the degranulation of mast cells *in vitro* [21,23], and the level of mMCP-1 in serum 30 min after the first challenge was increased in this IgE-sensitized model (Table 4), suggesting that the early response at the first challenge was IgE-dependent, probably through the activation of mast cells in the lungs. On the other hand, the fourth challenge-induced increase in sRaw at 10 min may have been influenced by not only IgE but also IgG1, because multiple antigen challenges in mice sensitized with IgE mAb enhanced the production of serum antigen-specific IgG1 (Fig. 3). In addition, the multiple antigen challenges increased the numbers of mast cells and basophils in the lung tissue (Nabe et al., unpublished data). Mechanisms underlying the early phase response produced after multiple antigen challenges remain to be clarified further.

Airway remodeling, including goblet cell hyperplasia and subepithelial fibrosis, is a characteristic of severe and/or chronic asthma [12–16]. Histological changes in the lung developed even in the IgE-sensitized model; however, the development of goblet cell hyperplasia was absent in IgE-sensitized SCID mice (Fig. 7), suggesting that goblet cell hyperplasia was dependent on IgE-mediated lymphocyte activation. Among these functional molecules, IL-4 and IL-13 have been reported to induce goblet cell hyperplasia *in vivo* [49,50]. In the present study, increases in the production of these cytokines in BALF of IgE-sensitized mice were observed (Fig. 8D), indicating that IL-13 as well as IL-4 is critical for the development of goblet cell hyperplasia in this IgE-sensitized model.

As for fibrotic changes, the seventh challenge induced subepithelial fibrosis in the lungs of IgE-sensitized mice, coinciding with the infiltration of eosinophils, although these responses were not significantly observed at the fourth challenge (Fig. 8B and F). Furthermore, the increase of IL-5 production in BALF, which is associated with the increase in the number of eosinophils, was observed at the seventh challenge, but not the fourth challenge (Fig. 8D). It has been reported that eosinophils are involved in antigen-induced subepithelial fibrosis by producing a fibrotic factor, transforming growth factor (TGF)-β1 [51]. In this IgE-sensitized

model, the increased expression of TGF-β1 was observed in the lung 24 h after the seventh challenge (data not shown). These findings suggest that the seven challenges in IgE-sensitized mice upregulate Th2-type lung inflammation, and that an increased number of eosinophils may participate in the exacerbation of subepithelial fibrosis through the production of TGF-β1 in the airway.

Collectively, in this study, we found that BALB/c mice sensitized with antigen-specific IgE mAb showed a biphasic asthmatic response, AHR, and airway remodeling after multiple antigen challenges. The allergic asthmatic responses induced by IgE mAb can be a useful model system to study the pathological roles of IgE in acute and chronic phases of allergic asthma *in vivo*. They may also be utilized to develop novel therapeutic strategies for allergic asthma.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgements

We are grateful to Dr. Kouya Yamaki (Kobe Pharmaceutical University) for establishing the hybridoma (OE-1), and Dr. Pilaiwanwadee Hutamekalin (Kobe Pharmaceutical University) for helpful comments. We thank Yasuhiro Ammori and Haruki Torii (Kobe Pharmaceutical University) for their assistance with measurements of samples.

Part of this work was supported by a Grant-in-aid for Young Scientists (B) (20790121) from the Ministry of Education, Science, Sport and Culture of Japan.

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