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Original Article

Inherent and antigen-induced airway hyperreactivity in NC mice

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Abstract

In order to clarify the airway physiology of NC mice, the following experiments were carried out. To investigate inherent airway reactivity, we compared tracheal reactivity to various chemical mediators in NC, BALB/c, C57BL/6 and A/J mice *in vitro*. NC mice showed significantly greater reactivity to acetylcholine than BALB/c and C57BL/6 mice and a reactivity comparable to that of A/J mice, which are known as high responders. Then, airway reactivity to acetylcholine was investigated in those strains *in vivo*. NC mice again showed comparable airway reactivity to that seen in A/J mice and a significantly greater reactivity than that seen in BALB/c and C57BL/6 mice. To investigate the effects of airway inflammation on airway reactivity to acetylcholine *in vivo*, NC and BALB/c mice were sensitized to and challenged with antigen. Sensitization to and challenge with antigen induced accumulation of inflammatory cells, especially eosinophils, in lung and increased airway reactivity in NC and BALB/c mice. These results indicate that NC mice exhibit inherent and antigen-induced airway hyperreactivity. Therefore, NC mice are a suitable strain to use in investigating the mechanisms underlying airway hyperreactivity and such studies will provide beneficial information for understanding the pathophysiology of asthma.

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Introduction

NC mice have biologically characteristic features, such as high susceptibility to X-irradiation, a high number of mast cells in the subcutaneous tissue and high susceptibility to anaphylactic shock from ovalbumin (OVA).[1–2] Recently, it has been reported that NC mice spontaneously develop symptoms that are histologically and clinically similar to human atopic dermatitis, with IgE production and increasing numbers of granulated mast cells in a conventional environment.[3] However, little is known about the airway reactivity of NC mice.

It is now well recognized that one of the characteristic features of asthma is airway hyperreactivity to nonspecific stimuli.[4] Airway reactivity is influenced by both genetic and environmental factors. There are several reports on genetic control of airway hyperreactivity in animals[5–8] and humans.[9–11] Environmental factors, such as antigen exposure[12–14] or activation of mast cells by anti-IgE[15] cause airway inflammation and lead to airway hyperreactivity in animal models.

In the present study, to clarify characteristics of airway reactivity in NC mice, inherent airway reactivity in NC, BALB/c, C57BL/6 and A/J mice was investigated *in vitro* and *in vivo*. It had been reported previously that C57BL/6 mice are low responders and A/J mice are high responders to acetylcholine and serotonin (5-HT) *in vivo*.[5] The effects of airway inflammation, caused by sensitization to and challenge with antigen, on airway reactivity to acetylcholine were also investigated in NC and BALB/c mice *in vivo*.

MATERIALS AND METHODS

Animals

BALB/c, C57BL/6 and A/J mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). NC mice were purchased from Clea Japan Inc. (Tokyo, Japan). Mice were housed under specific pathogen-free (SPF) conditions with free access to water and a standard laboratory diet.

Measurement of tracheal reactivity *in vitro*

Mice were killed and the trachea was carefully removed from each animal and transferred to a Petri dish filled with Tyrode solution (composition in mmol/L: NaCl 137.0, KCl 2.68, $CaCl_2$ 1.89, $MgCl_2$ 1.09, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, glucose 5.6, pH 7.4). The trachea was prepared free of excess tissue and cut into zig-zag strips according to the method of Akϵasu.[16] The tracheal segment preparations were mounted in an organ bath filled with 10 mL Tyrode solution and connected to a force-displacement transducer (AP-621G; Nihon Kohden Co., Tokyo, Japan) for registration of the isometric tension on a two-channel recorder (FBR-252A; TOA Electronics Ltd, Tokyo, Japan). The Tyrode solution was aerated with a mixture of oxygen (95%) and carbon dioxide (5%). Temperature was maintained at 37°C with a constant temperature circulating unit. The resting tension of the preparations was set at 0.5 g and preparations were allowed to equilibrate for 1 h. During the equilibration, the bath fluid was exchanged every 15 min. Test compounds were added in the tonic phase of the contraction. Results are presented as the percentage of the maximal contraction obtained in response to 50 mmol/L KCl.

Measurement of airway reactivity *in vivo*

In order to measure airway reactivity to acetylcholine, mice were anesthetized with sodium pentobarbitone (100 mg/kg; Dainabot Co., Osaka, Japan) by i.p. injection and the tracheas were surgically exposed and cannulated. The tracheal cannula was then connected to a rodent ventilator (Model 683; Harvard Apparatus, South Natick, MA, USA) and a bronchospasm transducer (Model 7020; Ugo Basile, Comerio-Varese, Italy). Mice were mechanically ventilated with air supplemented at 60 strokes/min, with a stroke volume of 0.6 mL. Bronchoconstriction was measured according to the overflow method described by Konzett and Rössler.[17] After administration of a paralytic agent (pancuronium bromide, 0.1 mg/kg; Sigma Chemical Co., St Louis, MO, USA), airway constriction was measured by determining changes in respiratory overflow volume during cumulative i.v. injection of acetylcholine. The increase in respiratory overflow volume provoked by acetylcholine was represented as a percentage of the maximal overflow (100%) obtained by clamping the trachea cannula. Area under the curve (AUC) was calculated from the results of the dose–response curve for acetylcholine.

Protocol of sensitization and inhalation challenge

The sensitization protocol conformed to the method described by Kung *et al.*[18] with slight modifications. Briefly, mice were actively sensitized by i.p. injection of 10 μg OVA with 1 mg alum on days 0 and 5. Then, mice were exposed to OVA (5 mg/mL in 0.9% NaCl solution) for 10 min on days 12, 16 and 20, by an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan). Twenty-four hours after the last antigen exposure, measurement of airway reactivity and collection of bronchoalveolar lavage fluid (BALF) were performed.

Collection of lung cells

Bronchoalveolar lavage fluid was collected by lavaging the whole lung via the tracheal cannula with 4×0.7 mL saline containing 0.1% bovine serum albumin (BSA) at room temperature. The BALF obtained from one animal was pooled, centrifuged and resuspended in 100 μL saline containing 0.1% BSA. Then, cell numbers were determined using a standard hemocytometer and 5×10^4 cells were spun onto glass slides and differentially stained by Diff-Quik (International Reagent Co., Kobe, Japan). Cell types were identified by morphological criteria. Two hundred cells were counted on each slide.

Measurement of serum IgE

Ovalbumin-specific IgE in sera was measured according to the enzyme-linked immunosorbent assay (ELISA) method described by Tomura *et al.*[19] with slight modifications. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 3 μg/mL rat anti-mouse IgE monoclonal antibody R35-72 (Pharmingen, San Diego, CA, USA). After blocking with SuperBlock

(Pierce, Rockford, IL, USA) and washing with Tris-buffered saline containing 0.05% Tween 20 (TBST), appropriate dilution of samples in TBST containing 1% BSA were added to the plate. After overnight incubation at 4°C, plates were washed and 1.4 μg/mL biotinylated OVA was added to each well. Biotinylation of OVA was performed using a biotinylation kit (Amersham Co., Arlington Heights, IL, USA). The plates were incubated for 1 h at room temperature and washed. Then, the binding of the antibody was measured using alkaline phosphatase-streptavidin conjugate (Zymed, San Francisco, CA, USA) and an alkaline phosphatase colormerizing kit (AMPAK; DAKO Japan Co., Kyoto, Japan) for amplification of the colorimetric signal. A serum pool from OVA-sensitized mice was used as an internal laboratory standard.

Statistics

Homogeneity of variance was tested by the *F*-test. The Student's *t*-test was applied in cases where the variance was homogeneous, and Aspin-Welch's test was performed when the variance was heterogeneous. A *P* value of less than 0.05 was considered significant. Values for all measurements are expressed as mean ± SEM.

RESULTS

Tracheal reactivity to acetylcholine, 5-HT and histamine in A/J, NC, BALB/c and C57BL/6 mice *in vitro*

Reactivity of the tracheal segment removed from each mouse to increasing doses of acetylcholine, 5-HT and

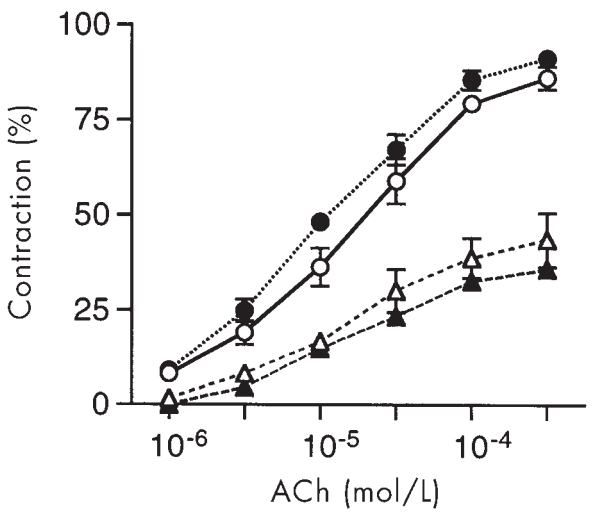

Fig. 1 Tracheal reactivity to acetylcholine (ACh) in various murine strains *in vitro*. Dose–response curves to ACh were measured in A/J (○, *n* = 4), NC (●, *n* = 5), BALB/c (△, *n* = 4) and C57BL/6 (▲, *n* = 4) mice. Results are expressed as the mean ± SEM.

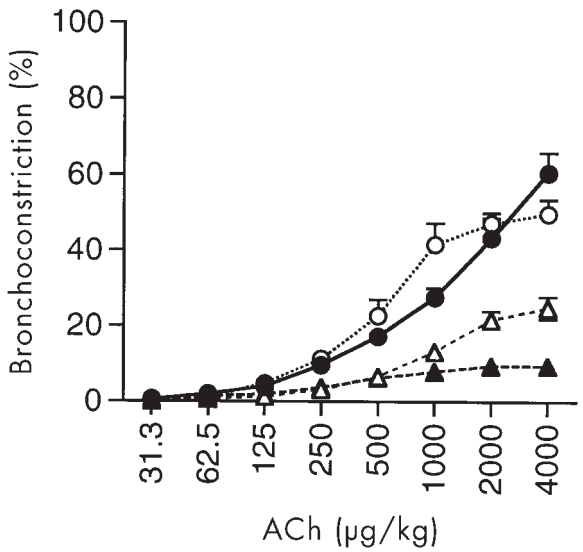

Fig. 2 Bronchial reactivity to acetylcholine (ACh) in various murine strains i*n vivo*. Dose–response curves to intravenous injection of ACh were measured in A/J (○), NC (●), BALB/c (△) and C57BL/6 (▲) mice. Results are expressed as the mean ± SEM. For each group, *n* = 6 mice.

Table 1 Comparison of tracheal reactivity to various chemical mediators between A/J, NC, BALB/c and C57BL/6 mice

	ACh (10^{-4} mol/L)	5-HT (10^{-4} mol/L)	Histamine (10^{-4} mol/L)
A/J	79.53 ± 1.38*†	13.85 ± 2.30	ND
NC	85.76 ± 2.55*†	18.48 ± 2.82‡	ND
BALB/c	38.78 ± 5.27	9.38 ± 1.05	ND
C57BL/6	32.60 ± 1.94	9.50 ± 2.10	ND

Results are presented as the percentage of maximal contraction obtained in response to 50 mmol/L KCl and are expressed as the mean ± SEM of four to five mice. ND, not detected.*$P < 0.001$ compared with C57BL/6 mice; †$P < 0.001$, ‡$P < 0.05$ compared with BALB/c mice. ACh, acetylcholine; 5-HT, serotonin.

histamine was measured. Dose–response curves for acetylcholine are shown in Fig. 1. NC mice showed greater reactivity to acetylcholine than BALB/c and C57BL/6 mice and reactivity comparable to that in A/J mice, which are known as high responders. Comparisons of tracheal reactivity to acetylcholine, 5-HT and histamine at a dose of 10^{-4} mol/L are summarized in Table 1. NC mice showed significantly greater reactivity to 5-HT than BALB/c mice. NC mice showed a greater tendency to respond to 5-HT than did C57BL/6 and A/J mice but the difference was not significant. In each strain, 5-HT produced less reactivity than acetylcholine. BALB/c and C57BL/6 mice showed comparable airway reactivity to acetylcholine and to 5-HT (Fig. 1; Table 1). None of the strains responded to histamine up to 3×10^{-4} mol/L. Leukotriene C_4 and platelet-activating factor did not provoke tracheal contraction up to 10^{-9} mol/L and 3×10^{-6} mol/L, respectively.

Table 2 Comparison of area under the curve values between A/J, NC, BALB/c and C57BL/6 mice

Strain	AUC
A/J	46.17 ± 4.78*†
NC	40.29 ± 2.99*†
BALB/c	17.59 ± 1.74†
C57BL/6	10.52 ± 0.51

Values of the area under the curve (AUC) were obtained from the dose–response curves shown in Fig. 2. Values are expressed as the mean ± SEM of six mice. *$P < 0.01$ compared with BALB/c mice; †$P < 0.01$ compared with C57BL/6 mice.

Airway reactivity to acetylcholine in A/J, NC, BALB/c and C57BL/6 mice in vivo

Bronchoconstriction provoked by intravenous injection of increasing doses of acetylcholine was measured. Dose–response curves are shown in Fig. 2. At a dose of 4000 μg/kg, NC, A/J, BALB/c and C57BL/6 mice showed 60.72%, 49.86%, 24.72% and 9.36% of maximal constriction, respectively. Values of AUC obtained from dose–response curves are summarized in Table 2. Values of AUC revealed that NC mice showed greater reactivity than BALB/c and C57BL/6 mice and reactivity comparable to that of A/J mice. BALB/c mice showed greater reactivity than C57BL/6 mice.

Antibody responses to OVA sensitization and cellular composition of BALF in BALB/c and NC mice

BALB/c and NC mice were actively sensitized to and challenged with OVA. Peripheral blood samples were

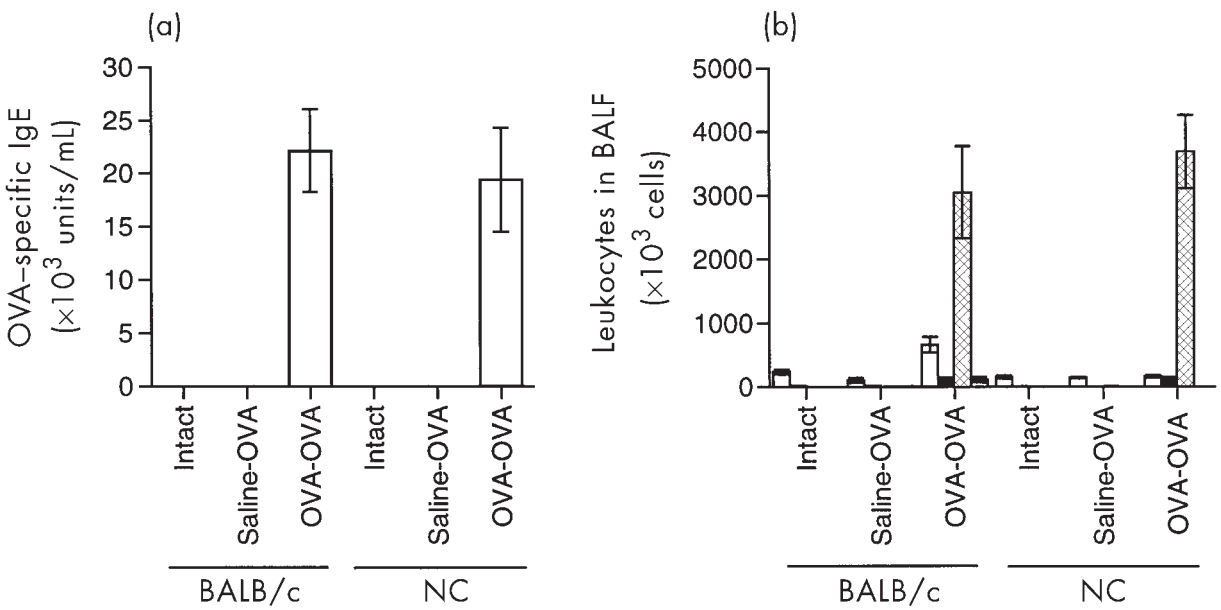

Fig. 3 (a) Serum levels of ovalbumin (OVA)-specific IgE antibodies and (b) cellular composition of bronchoalveolar lavage fluid (BALF) in BALB/c and NC mice (□, macrophages; ■, lymphocytes; ▩, eosinophils; ▨, neutrophils). Ovalbumin-specific IgE antibodies and cellular composition of BALF were determined in mice after sensitization and challenge ($n = 7$), mice receiving challenge alone ($n = 6$) and intact mice ($n = 6$). Results are expressed as the mean ± SEM for each group. Saline-OVA, challenge alone; OVA-OVA, sensitized and challenged.

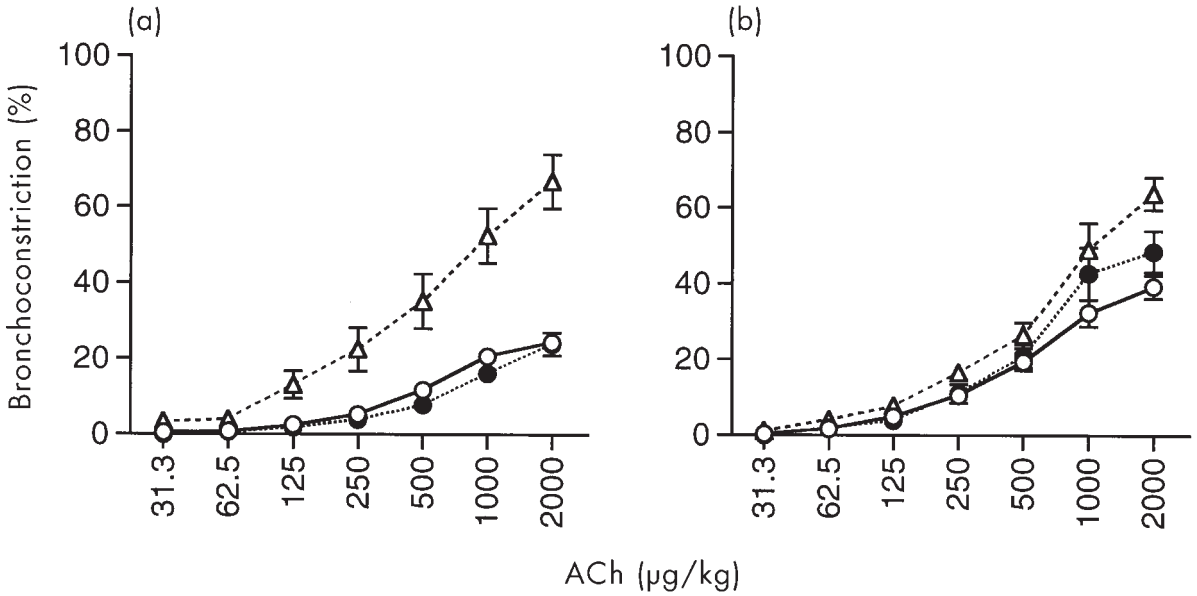

Fig. 4 Bronchial reactivity to acetylcholine (ACh) in (a) BALB/c and (b) NC mice *in vivo*. Dose–response curves to intravenous injection of ACh were measured in mice after sensitization and challenge (△; $n = 7$), mice receiving challenge alone (●; $n = 6$) and intact mice (○; $n = 6$). Results are expressed as the mean ± SEM for each group.

Table 3 Comparison of area under the curve values in BALB/c and NC mice

Strain	Group	AUC
BALB/c	Intact	15.97 ± 1.18
BALB/c	Saline-OVA	12.63 ± 1.12
BALB/c	OVA-OVA	48.7 ± 7.84*†
NC	Intact	26.66 ± 2.29
NC	Saline-OVA	31.53 ± 4.50
NC	OVA-OVA	40.98 ± 3.37*

Values of the area under the curve (AUC) were obtained from the dose–response curves shown in Fig. 4. Values are expressed as the mean ± SEM of six to seven mice. Statistical analysis was performed separately for each strain, between groups. * $P < 0.01$ compared with the intact group; † $P < 0.01$ compared with the saline-ovalbumin (OVA) group.

collected from the retro-orbital sinus at day 20. Active sensitization to and challenge with OVA caused elevation of serum levels of OVA-specific IgE in both strains. The increasing levels were comparable in BALB/c and NC mice (Fig. 3a). For both strains, neither intact mice, which did not receive either sensitization or challenge, nor mice receiving challenge alone showed an IgE response.

Leukocyte accumulation in BALF is shown in Fig. 3(b). In intact mice and mice receiving challenge alone, macrophages were the predominant cell type accumulated in BALF. In contrast, sensitization and challenge caused accumulation predominantly of eosinophils, with a relatively low accumulation of lymphocytes and neutrophils in both strains. The number of lymphocytes, eosinophils and neutrophils in sensitized and challenged groups were comparable for both strains.

Antigen-induced airway hyperreactivity to acetylcholine in BALB/c and NC mice

Changes in airway reactivity to acetylcholine by sensitization to and challenge with antigen in BALB/c and NC mice were investigated. Dose–response curves for acetylcholine are shown in Fig. 4. In this experiment, again, intact NC mice showed inherent airway hyperreactivity (Fig. 4b) when compared with intact BALB/c mice (Fig. 4a). Accumulation of inflammatory cells in the peribronchial tissue and histological lesion was not observed in intact NC mice (data not shown). The AUC values obtained from dose–response curves are summarized in Table 3. In BALB/c mice, the intact group and mice receiving challenge alone showed comparable airway reactivity. Airway hyperreactivity was observed in mice receiving both sensitization and challenge. In NC mice, mice receiving sensitization and challenge had increased airway reactivity compared with the intact group ($P < 0.01$). Mice receiving challenge alone showed a tendency toward increased airway reactivity compared with the intact group, but the difference was not significant.

Discussion

Airway hyperreactivity is one of the characteristic features of asthma.[4] Airway reactivity is influenced by genetic factors. In inbred strains of mice, there is a wide variability in inherent airway reactivity between strains.[5–7,20] Pauwels *et al.* showed similar variability of inherent airway reactivity among inbred strains of rats.[21] In the present study, we demonstrated that NC mice, in the absence of inflammation, showed high airway reactivity to acetylcholine and 5-HT *in vitro* (Fig. 1; Table 1). *In vivo* experiments revealed that NC mice also showed high airway reactivity to acetylcholine comparable to that in A/J mice, which are known as high responders (Fig. 2; Table 2). In contrast, C57BL/6 and BALB/c mice showed low airway reactivity to acetylcholine *in vitro* and *in vivo.*

Levitt and Mitzner reported that A/J mice showed high reactivity to acetylcholine, while BALB/c and C57BL/6 showed low reactivity to acetylcholine *in vivo*.[5] Our data support those *in vivo* findings. Furthermore, we showed that those *in vivo* findings were consistent with our results *in vitro*. We also demonstrated that none of the strains responded to histamine, while acetylcholine and 5-HT were reactive *in vitro*. These results were consistent with the previous *in vivo* findings that histamine does not cause bronchoconstriction while acetylcholine and 5-HT provoke bronchoconstriction in C57BL/6 mice.[22]

De Sanctis *et al.* demonstrated that airway reactivity is a heritable trait, which is inherited as a dominant trait and influenced by at least three loci that map to chromosomes 2, 15 and 17 in mice.[6] These loci include several candidate genes that may control airway reactivity. They also demonstrated that T lymphoctyes were involved in the regulation of inherent airway hyperreactivity.[23] Nicolaides *et al.* reported that the interleukin (IL)-9 gene is a candidate gene for regulating inherent airway reactivity.[24] Further experiments are required to determine the cell(s) and/or gene(s) responsible for the regulation of inherent airway hyperreactivity in NC mice. Analysis of differences in sequences and expression patterns of candidate genes and differences in the function of T lymphocytes between hyperreactive and hyporeactive strains will provide beneficial information for understanding the mechanisms underlying the pathophysiology of asthma. As a hyperreactive strain, the NC murine strain, together with the A/J strain, is suitable for use in such experiments.

Airway reactivity is also considered to be influenced by environmental factors. It has been reported that airway inflammation caused by antigen exposure[12–14] or activation of mast cells by anti-IgE[15] leads to airway hyperreactivity in animal models. We therefore investigated the effects of sensitization and challenge with antigen on airway reactivity to acetylcholine in NC and BALB/c mice *in vivo*. Under SPF conditions, we could not detect any IgE in intact NC mice (data not shown), which coincided with a previous finding.[3] Active sensitization by OVA via the peritoneal cavity resulted in production of IgE in both strains under SPF conditions. The levels of IgE production were comparable in NC and BALB/c mice (Fig. 3a). Matsuda *et al.* reported that in NC mice the levels of total IgE spontaneously increased under conventional conditions, but did not increase in BALB/c mice under the same conditions.[3] These differences may reflect the high penetration of antigen via the skin in NC mice.

Sensitization and challenge with OVA induced dominant accumulation of eosinophils with a small number of lymphocytes and neutrophils in BALF, but such induction was not shown in mice receiving challenge alone (Fig. 3b). The numbers of those inflammatory cells in BALF were comparable in NC and BALB/c mice. The number of eosinophils observed in BALF was relatively high in comparison with previous reports.[12,14,18] In our protocol of sensitization and challenge, dominant accumulation of eosinophils is observed reproducibly. Dose of antigen, timing of secondary sensitization and condition of challenge may influence the number of eosinophils accumulated in lung. Airway reactivity of NC mice was increased in the sensitized and challenged group (compare the OVA-OVA group with the intact group in Table 3), but the increase was relatively small when compared with BALB/c mice. In the sensitized and challenged group, NC and BALB/c mice exhibited comparable bronchoconstriction to acetylcholine at a dose of 2000 μg/kg (63.86 ± 4.33% and 66.87 ± 7.19%, respectively). NC mice showed greater airway reactivity than BALB/c mice in the intact group, which may be one of the reasons that NC mice in the sensitized group exhibited a relatively small increase in airway reactivity following antigen challenge. The role of eosinophils in the development of airway hyperreactivity is controversial in murine models of antigen-induced airway hyperreactivity.[13,25,26] Although a high number of eosinophils was observed in our model, the role of eosinophils is still unclear. Further experiments are required to determine the role of inflammatory cells, such as eosinophils, mast cells and T lymphocytes, and possible molecule(s) in our model of antigen-induced airway hyperreactivity.

In conclusion, we have demonstrated that NC mice show inherent airway hyperreactivity *in vitro* and *in vivo*. Active sensitization to and challenge with antigen increased airway reactivity to acetylcholine when compared with an intact group of NC mice. These results suggest that NC mice are a suitable strain for investigation of the mechanisms of airway hyperreactivity.

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