Dissociation between airway responsiveness to methacholine and responsiveness to antigen

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Dissociation between airway responsiveness to methacholine and responsiveness to antigen. A. Kamachi, Y. Nasuhara, M. Nishimura, T. Takahashi, Y. Homma, Y. Ohtsuka, M. Munakata. ©ERS Journals Ltd 2002.

ABSTRACT: Repeated aerosolized antigen challenges to brown Norway (BN) rats generate nonspecific airway hyperresponsiveness (AHR). On the other hand, some studies have demonstrated that repeated antigen challenge could attenuate antigen-specific AHR in BN rats. The authors questioned whether such dissociation in airway responses actually occurs when assessed in a single study in the same animals.

The authors simultaneously measured AHR to methacholine and antigen-specific AHR in rats that were repeatedly exposed to aerosolized ovalbumin (OA) for 1 or 3 months after sensitization. Four days after the last challenge, airway responses to methacholine and OA, morphometry of the airways, the cell profile in bronchoalveolar lavage fluid, and cytokine messenger ribonucleic acid (mRNA) expression in the lungs were evaluated.

The two types of AHR were modulated in opposite directions by repeated antigen challenges. The AHR to methacholine was significantly increased in the rats receiving antigen challenges compared with the control rats receiving saline challenges after sensitization; whereas, the antigen-specific AHR was significantly decreased. The number of alveolar macrophages in lavaged fluid and the expression of transforming growth factor- β_1 mRNA in lung tissue was significantly different between the antigen-challenged rats and the control rats.

In conclusion, dissociation between nonspecific airway hyperresponsiveness and antigen-specific airway hyperresponsiveness in brown Norway rats after repeated antigen challenges was demonstrated. Sustained airway inflammation with macrophages and/or upregulation of transforming growth factor- β_1 messenger ribonucleic acid in the lung tissue may be responsible for this dissociation.

Eur Respir J 2002; 19: 76–83.

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Keywords: Airway hyperresponsiveness
macrophage
repeated antigen challenge
transforming growth factor- β

Received: January 29 2001

Accepted after revision October 3 2001

Brown Norway (BN) rats have been used as a model of atopic asthma [1–3]. Sensitization and a subsequent single antigen challenge to these rats induce a number of immunological, physiological and pathological features similar to those observed in human allergic asthma. These include production of an antigen-specific immunoglobulin-E (IgE) antibody [1], upregulation of T-helper 2 cytokines such as interleukin (IL)-4 and/or IL-5 [2], early and late phase airway reactions to the inhaled antigen [1], eosinophilic airway inflammation, and nonspecific airway hyperresponsiveness (AHR) to methacholine [3]. In the same strain of rats, multiple antigen challenges are reported by some investigators to develop chronic abnormalities resembling those in asthma, such as airway remodelling [4, 5]. In addition, it has been demonstrated that nonspecific AHR occurs after multiple antigen challenges in some reports [4–8]. In contrast, there have been a number of studies, which observed the generation of IgE isotype-specific tolerance [9, 10] after multiple antigen challenges and also the attenuation of antigen-specific AHR.

Thus, the present authors were interested in

the possible dissociation of nonspecific AHR from antigen-specific AHR in repeatedly antigen-challenged BN rats. The authors then attempted to examine the mechanism by which nonspecific AHR was changed in such desensitized animals. To achieve this goal, morphological changes of the airways, inflammatory cells in bronchoalveolar lavage fluid (BALF), and cytokine messenger ribonucleic acid (mRNA) expression in the lungs, which are considered to be the factors associated to the development of nonspecific AHR, were examined. It was anticipated that this study might provide insights into the reason why nonspecific AHR could remain increased under the condition of desensitization.

Materials and methods

Immunization and airway sensitization

All experimental protocols and procedures were approved by the Ethical Committee on Animal

Research, Hokkaido University School of Medicine, Sapporo, Japan. Specific pathogen-free 6-week-old male BN rats (weight range 160–210 g) were purchased from Japan Charles River Co. (Yokohama, Japan). The rats were kept in a conventional colony in a Hokkaido University animal care facility. They were actively sensitized for ovalbumin (OA) by subcutaneous injection with 1 mg of OA dissolved in gel including 200 mg of aluminium hydroxide. An adjuvant (1 mL) consisting of 1×10^9 heat-killed *Bordetella pertussis* organisms was injected intraperitoneally at the same time. Two weeks after the sensitization, the rats inhaled 2% weight/volume OA for 15 min in an exposure chamber (35×25×20 cm) to sensitize the airway. For inhalation challenge, OA was dissolved in saline and aerosolized with an ultrasonic nebulizer (NE-U11B, Omuron Co., Tokyo, Japan), which delivered the aerosol at $1 \text{ mL} \cdot \text{min}^{-1}$. For repeated antigen exposure, 5% OA was challenged for 10 min.

Experimental protocol

Rats receiving a single OA challenge alone (single-OA, $n=5$) were examined 4 days after the challenge to confirm increased airway responses to methacholine (Mch) and OA. For comparison, sham-sensitized and saline-challenged rats (single-sham, $n=4$) were also examined. To examine the effects of repeated antigen challenges, the other single-OA rats were divided into two groups, and they received further challenges with either OA or saline. One group of rats received repeated OA challenges every 2 days for 4 weeks (1M-OA, $n=8$) or 12 weeks (3M-OA, $n=8$). The other group received saline instead of OA in an identical way (1M-cont, $n=8$; 3M-cont, $n=8$).

Measurement of bronchial responsiveness to methacholine and ovalbumin

Four days after the final challenge, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium ($50 \text{ mg} \cdot \text{kg}^{-1}$). Intratracheal intubation was then performed with a metallic tube (2 mm internal diameter). The rats were mechanically ventilated (Rodent Ventilator Model 683, Harvard Apparatus, Holliston, MA, USA) with a tidal volume of $6 \text{ mL} \cdot \text{kg}^{-1}$ and frequency of $100 \text{ breaths} \cdot \text{min}^{-1}$. A pressure transducer (TP-602T, Nihon Kohden Co., Japan) was connected to a side port of the metallic tube, and airway opening pressure (P_{ao}) was continuously measured. An aerosol of Mch or OA was produced using an ultrasonic nebulizer with an output of $0.2 \text{ mL} \cdot \text{min}^{-1}$ and administered through a reservoir box connected to the ventilator system. After measurement of baseline P_{ao} , an aerosol of saline followed by Mch was administered for 1 min in progressively doubled concentrations from $0.0625 \text{ mg} \cdot \text{mL}^{-1}$. The airway response to Mch was assessed by the Mch concentration needed to induce a 50% increase from baseline P_{ao} (PC150Mch).

After the Mch inhalation test, the rats were forced to take deep breaths every 2 min for >15 min until

P_{ao} returned to near the baseline level. Five per cent OA was then inhaled for 10 min. The airway response to OA was expressed as the per cent increase of P_{ao} from before to after OA inhalation.

Morphometric dimensions

Immediately after the measurement of airway responsiveness, the rats were exsanguinated from the aorta, and the left lung was fixed with an intrabronchial infusion of 10% neutral formalin at a constant pressure of $25 \text{ cmH}_2\text{O}$ for a period of 48 h. Paraffin-embedded midsagittal sections (5 μm thick) were then prepared and stained with haematoxylin and eosin in order to evaluate the density of eosinophils in the airways. Airway smooth muscle was stained with a monoclonal anti- α smooth muscle actin antibody (clone 1A4; DAKO Danmark A/S, Glostrup, Denmark) by the avidin-biotin alkaline phosphatase complex method (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA, USA). Vector red (Vector Laboratories), which is highly fluorescent, was used as the substrate for alkaline phosphatase to differentiate airway smooth muscle from the surrounding connective tissue.

An image analyser (XL-10, Olympus Co., Tokyo, Japan) was used in combination with light/fluorescent microscopy. Only airways with a short-to-long diameter ratio of ≤ 0.5 were analysed. Approximately 10 airways were measured for each rat. The internal and external perimeter (equal to the length of the basement membrane) of the epithelium, and the external perimeter of the airway wall were traced. The area of airway smooth muscle was measured after changing the microscopic view from light to fluorescence. Airways were divided into three groups by the length of the basement membrane (BM) (S: 0.5–1 mm; M: 1–2 mm; L: ≥ 2 mm). All areas were divided by the BM length² to standardize the variation of airway size. Areas of airway smooth muscle, airway wall and epithelium were compared in the same-sized airways between the groups. Morphometry was evaluated by an independent researcher, who was unaware of the source of specimens at the time of evaluation.

Bronchoalveolar lavage and cell counting

Another set of six groups (single-sham, single-OA, 1M-cont, 1M-OA, 3M-cont, 3M-OA) was prepared to examine the profile of inflammatory cells in BALF and cytokine mRNA expression in the lungs.

Four days after the final aerosol challenge, the lungs were lavaged four times with a total volume of 20 mL of sterile saline, while a 16-gauge catheter was placed into the trachea. After the lavage, the right lungs were frozen for later studies of cytokine mRNA expression. The lavage fluid was centrifuged (5 min, $500 \times g$ at 4°C), and the cells were resuspended in 1 mL of Hanks' balanced solution (Cosmo Bio Co. Ltd, Tokyo, Japan). Cells were counted and processed for differential cell analysis using Giemsa staining by counting 300 cells. Flow cytometry was performed to count the

number of CD4 and CD8 lymphocytes. The 5×10^5 cells in lavage fluid were stained with a fluorescein isothiocyanate-conjugated mouse antirat CD4 (OX-38) or CD8 (OX-8) monoclonal antibody (Pharmingen, San Diego, CA, USA) for 30 min at 4°C. After washing with phosphate-buffered saline, flow cytometry was performed by fluorescence-activated cells sorting (FACS) using a FACScan analyser (Becton Dickinson FACS Division, Sunnyvale, CA, USA).

Real-time quantitative reverse transcriptase polymerase chain reaction assay for interleukin-13, interferon- γ , tumour necrosis factor- α and transforming growth factor- β_1 messenger ribonucleic acid

Total lung ribonucleic acid was extracted from frozen lungs using a commercial kit (ISOGEN, Nippon Gene Co., Toyama, Japan) according to the standard procedure [11]. A two-step reverse transcriptase polymerase chain reaction (RT-PCR) procedure was used according to the protocol of the *TaqMan* Gold RT-PCR Kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The primers and probes for rat IL-13, interferon (IFN)- γ , tumour necrosis factor (TNF)- α and transforming growth factor (TGF)- β_1 were defined using primer express software (Perkin-Elmer Applied Biosystems) (table 1). As a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also amplified at the same time using *TaqMan* rodent GAPDH control

reagent (Perkin-Elmer Applied Biosystems). Reverse transcription was performed at 48°C for 30 min. Polymerase chain reaction (PCR) amplification and simultaneous quantification of a target sequence were then carried out using the PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems). PCR was performed for 10 min at 95°C, followed by 40 cycles of amplification (95°C for 15 s and 60°C for 90 s) for all samples in duplicate. Details of this method are described in previous reports [12, 13]. The absolute number of transcript copies was normalized to an endogenous control, the GAPDH transcript. Results were expressed as a relative ratio for the mean level of the single-sham group.

Statistical analysis

Because the results were not normally distributed, the OA-challenged groups were compared with the control groups by the nonparametric Mann-Whitney U-test. A difference was considered to be significant when $p < 0.05$. All results in the text, tables and figures are presented as mean $\pm$ SEM.

Results

Airway responsiveness to methacholine and ovalbumin

Airway responsiveness either to Mch or to OA was significantly increased in the single-OA group compared with the single-sham group (figs. 1 and 2). Both log PC_{150Mch} and the % increase of P_{ao} gradually declined over time, when the rats received repeated saline challenges. However, repeated antigen challenges significantly attenuated the time-dependent decline in log PC_{150Mch}, so that both 1M-OA and 3M-OA groups showed significantly higher non-specific AHR than the control groups. By contrast, repeated antigen challenges significantly accelerated

Table 1.—Primers and probes used for real-time quantitative reverse transcriptase polymerase chain reaction

IL-13		
F-primer	5'CCTGGAATCCCT	GACCAACAT3'
R-primer	5'CAGAGGCCATTC	AATATCCTCTG3'
Probe	5'TCCAGTTGCAATG	CCATCCACAGGAC3'
IFN- γ		
F-primer	5'ACCAGCTGTCACC	AGAATCTAGC3'
R-primer	5'TGGCACACTCTC	TACCCAGAA3'
Probe	5'TAAGGAAGCGAAAA	GGAGTCGGTGCTG3'
TNF- α		
F-primer	5'GCTCCCTCTCAT	CAGTTCCATG3'
R-primer	5'TTGGTGGTTTGC	TACGACGTG3'
Probe	5'CCCAGACCCTCACACT	CAGATCATCTTCT3'
TGF- β_1		
F-primer	5'GCTTCAGCTCCA	CAGAGAAGAA3'
R-primer	5'TCCAACCCAGG	TCCTTCCTAA3'
Probe	5'TGCTGTGTACGGCAG	CTGTACATTGAC3'

IL: interleukin; IFN: interferon; TNF: tumour necrosis factor; TGF: transforming growth factor; F: forward; R: reverse.

Fig. 1.—Airway responsiveness to methacholine (Mch) after single, 1-month and 3-month antigen challenges in rats. PC_{150Mch}: Mch concentration needed to induce a 50% increase from baseline airway opening pressure. □: unsensitized sham controls in the singly-challenged group; ○: sensitized and saline-challenged controls; ●: sensitized and ovalbumin-challenged groups. Data are presented as mean $\pm$ SEM. *: $p < 0.05$.

Fig. 2.—Airway responsiveness to ovalbumin (OA) after single, 1-month and 3-month antigen challenges in rats. Data are expressed as percentages of increased airway opening pressure (P_{ao}) (after/before OA inhalation for 10 min). □: unsensitized sham controls in the singly-challenged group; ○: sensitized and saline-challenged controls; ●: sensitized and OA-challenged groups. Data are presented as mean±SEM. *: $p < 0.05$.

the time-dependent decline in the % increase of P_{ao} after antigen inhalation, so that both 1M-OA and 3M-OA groups showed significantly lower antigen-specific AHR than the control groups.

Morphometrical analysis

A significant difference was noted between the 1M-OA group and the 1M-control group, only in the

smooth muscle as well as the walls and epithelia of the small airways (fig. 3). Despite such positive effects of a 1-month OA challenge on airway morphology, there were no significant differences in any parameters of airway morphology between the 3M-OA group and the 3M-control group.

Total and differential cell counts of lavaged cells

In the single-OA group, there was a six-fold increase in the total cell number compared with the single-sham group (table 2). Differential cell counts revealed that alveolar macrophages and eosinophils were generally responsible for the increase in total cell number. After repeated antigen or saline challenges, however, there were no significant differences between the two groups in the number of eosinophils, because eosinophils disappeared whether the rats received repeated antigen challenges or not. A significant difference was noted between the two groups in the number of alveolar macrophages (*i.e.* both 1M-OA and 3M-OA groups showed significantly higher numbers of alveolar macrophages than the control groups).

The numbers of CD4+ and CD8+ lymphocytes were also significantly increased in the single-OA and 1M-OA groups compared with the control groups. However, there were no significant differences in these cell counts between the repeatedly antigen-challenged rats and the control rats at 3 months (table 3).

Fig. 3.—Morphometrical dimensions of the airways after single (a, d, g), 1-month (b, e, h) and 3-month (c, f, i) antigen challenges in rats. Y-axes show area collected by airway size (area divided by the length² of the basement membrane): a–c) smooth muscle; d–f) wall; g–i) epithelium. S: small airways; M: middle-sized airways; L: large airways. □: unsensitized sham controls in the singly-challenged group; ▤: sensitized and saline-challenged controls in repeatedly-challenged groups; ▨: sensitized and ovalbumin-challenged groups. Data are presented as mean±SEM. *: $p < 0.05$.

Table 2. – Total and differential cell counts in bronchoalveolar lavage fluid (BALF)

Group	n	Total cells	MΦ	Lymph	Eosinophils	Neutrophils
Single-sham	8	88±19	78±17	0.5±0.2	9.6±5.7	0.09±0.05
Single-OA	8	520±47***	332±32***	18.4±4.9***	169.5±33.1***	0.00±0.00
1M-cont	8	143±12	140±12	1.4±0.4	1.6±0.3	0.48±0.16
1M-OA	8	353±43 [#]	344±42 [#]	6.4±1.2 [#]	2.1±0.5	0.25±0.18
3M-cont	8	183±16	176±15	3.8±0.7	2.4±0.7	1.65±0.54
3M-OA	8	309±25**	298±26**	5.7±1.2	3.9±1.1	0.20±0.20*

Data are presented as mean±SEM and number of cells ($\times 10^4$) in 1 mL of BALF; OA: ovalbumin; 1M: 1 month; 3M: 3 months; cont: control. *: $p < 0.05$ compared with 3M-cont; **: $p < 0.01$; #: $p < 0.01$ compared with 1M-cont; ***: $p < 0.001$ compared with single-sham.

Expression of messenger ribonucleic acid for interleukin-13, interferon- γ , tumour necrosis factor- α and transforming growth factor- β_1

Only the level of TGF- β_1 mRNA among the four cytokines examined was significantly upregulated in the 3M-OA compared with the 3M-control group (fig. 4).

Discussion

In the present study, the authors demonstrated dissociation between nonspecific AHR and antigen-specific AHR after repeated antigen challenges in BN rats. The two types of AHR were modulated in opposite directions by repeated antigen challenges in rats sensitized with ovalbumin. The time-dependent decline in antigen-specific AHR was significantly accelerated by repeated antigen challenges, indicating the occurrence of desensitization. In contrast, the natural time-dependent decline in nonspecific AHR after one-time sensitization was significantly attenuated by repeated antigen challenges. The number of alveolar macrophages in BALF and the expression of TGF- β_1 mRNA were the only factors examined in the present study that might explain the dissociation of the two AHRs with repeated antigen challenges.

A number of previous studies demonstrated that multiple antigen challenges generated a marked increase in nonspecific AHR in BN rats [6–8]. On the other hand, several studies examining the immunological aspects of multiple antigen challenge demonstrated in the same strain of rats that such

challenges might induce IgE isotype-specific tolerance [9, 10]. Tolerance to a specific antigen should result in the suppression of eosinophilic inflammation of the airways, and thus, also the suppression of antigen-specific AHR. Accordingly, the authors attempted to prove the dissociation of nonspecific AHR from antigen-specific AHR in the same group of rats. To the authors' best knowledge, this is the first study providing evidence that such dissociation really occurs in the same animals.

An attempt was made to examine the mechanism underlying the dissociation. To study the profile of inflammatory cells in BALF would explain which cell type was involved in the enhancement of nonspecific AHR and/or the suppression of antigen-specific AHR with repeated antigen challenges. In the present study, alveolar macrophages were the only cell type that was significantly increased in rats receiving repeated antigen challenges, compared with the control rats receiving only saline instead. It is well known that macrophages have the potential to release pro-inflammatory mediators, including leukotriene B₄, platelet activating factor and nitric oxide, which have an important role on bronchial hyperresponsiveness [14, 15]. Thus, it can be speculated that airway inflammation induced by macrophages may play a role in the enhancement of nonspecific AHR in the present study's model rats receiving multiple antigen challenges. This speculation is supported by a human study that demonstrated a correlation of nonspecific AHR with the number of alveolar macrophages in BALF in asthmatic children [16]. On the other hand, some evidence is available suggesting that macrophages are also involved in inducing immunological tolerance [17, 18]. Accordingly, an increase in the number of alveolar macrophages may also have contributed to the suppression of antigen-specific AHR in the present experiment. By contrast, eosinophilic inflammation of the airways, elicited after the single antigen challenge, completely disappeared after repeated antigen challenges. This indicated that eosinophils were not responsible for the sustained increase in nonspecific AHR in the rats receiving repeated antigen challenges.

Another mechanism that may account for enhanced nonspecific AHR with repeated antigen challenges is morphological changes of the airways. In asthma, thickening of airway smooth muscles may lead to narrowing of the airways [19], and thus contribute to

Table 3. – Number of CD4⁺ and CD8⁺ lymphocytes

Group	n	CD4	CD8
Single-sham	8	1.9±0.9	0.3±0.1
Single-OA	8	124.5±30.7***	17.7±7.9***
1M-cont	8	7.7±3.0	1.0±0.3
1M-OA	8	28.1±5.2 [#]	10.1±2.0 [#]
3M-cont	8	23.6±4.5	5.0±0.9
3M-OA	8	30.7±7.1	7.9±2.0

Data are presented as mean±SEM and number of CD4⁺ and CD8⁺ ($\times 10^3$) in 1 mL of bronchoalveolar lavage fluid. OA: ovalbumin; 1M: 1 month; 3M: 3 months; cont: control. ***: $p < 0.001$ compared with single-sham; #: $p < 0.001$ compared with 1M-cont.

Fig. 4.—Expression of messenger ribonucleic acid for a) interleukin (IL)-13, b) tumour necrosis factor (TNF)- α , c) interferon (IFN)- γ , and d) transforming growth factor (TGF)- β_1 after single and repeated antigen challenges in rats. Y-axes show relative ratios to the single-sham group. $\square$: unsensitized sham controls in the singly-challenged group; $\circ$: sensitized and saline-challenged controls in repeatedly-challenged groups; $\bullet$: sensitized and ovalbumin (OA)-challenged rats. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; cont: control; 1M: 1 month; 3M: 3 months. Data are presented as mean $\pm$ SEM. *: $p < 0.05$.

nonspecific AHR. This mechanism was postulated to be true in previous experiments, which used BN rats treated in a way similar to the present experiment [4, 5]. Contrary to the present authors' expectation, however, the morphological changes of the airways observed after 1 month of antigen challenges disappeared after 3-month repeated antigen challenges; and there were no significant differences in the morphology of the airways between the repeatedly antigen-challenged rats and the control rats. The reason why it was not possible to develop airway remodelling may be explained by differences in the experimental protocol. The present authors used a larger amount of inhaled antigen, and/or gave it to the animals more frequently, for a relatively longer period than in previously published reports. However, the possibility of any contribution of airway remodelling to the enhancement of nonspecific AHR cannot be completely denied, because there may have been morphological changes in the airways smaller than those examined in the present study.

Previous studies suggested that cytokines such as IL-13, IFN- γ and TNF- α are involved in the development of nonspecific AHR in murine models of atopic asthma [20–23]. TGF- β_1 is a growth factor that is believed to play an important role in the development of airway remodelling in asthma, thus

contributing to the development of nonspecific AHR [24]. TGF- β_1 , on the other hand, was reported to suppress nonspecific AHR in a murine model of atopic asthma [25], and it may also be responsible for the development of immunological tolerance [25, 26]. Accordingly, TGF- β_1 is a potential candidate accounting for the dissociation of nonspecific AHR from antigen-specific AHR. Indeed, the present authors found that only the level of TGF- β_1 mRNA was significantly upregulated among the four cytokines examined for the whole lung tissue in rats receiving repeated antigen challenges compared to controls. Although it remains to be clarified whether upregulation of TGF- β_1 mRNA is really linked to the enhancement of nonspecific AHR with repeated antigen challenges, this cytokine may prove to play a key role in the dissociation of nonspecific AHR from antigen-specific AHR.

As discussed previously, it appears that a number of factors are involved in the development of nonspecific AHR. It is highly likely that the nonspecific AHR in the inflammatory airways depends on the balance of the protective and worsening factors. Thus, the authors feel that the present study actually reflects such complexity of the development of nonspecific AHR.

Antigen-specific immunotherapy is a strategy for

the treatment of allergic asthma in children. However, the efficacy of immunotherapy has not been universally accepted. The National Institutes of Health (NIH) International Consensus Report on Diagnosis and Management and the World Health Organization (WHO)/NIH Global Initiative on Asthma Management recommend that allergen immunotherapy should only be considered when asthma is poorly controlled with drugs, and where allergen avoidance is impossible [27, 28]. There has been at least one report, MURRAY *et al.* [29], which demonstrated the enhancement of nonspecific AHR by more than two-fold after mite immunotherapy in patients with atopic asthma. Although it may be premature to apply the results of the present study directly to humans, the animal model presented in this report may help to clarify why allergen immunotherapy is not always as effective as expected.

In conclusion, the authors have demonstrated a dissociation of airway hyperresponsiveness to methacholine from antigen-specific airway hyperresponsiveness in brown Norway rats that received repeated antigen challenges after ovalbumin sensitization. Sustained airway inflammation with macrophages and/or upregulation of transforming growth factor- β_1 messenger ribonucleic acid in the lung tissue may be responsible for this dissociation.

Acknowledgements. The authors would like to thank Y. Kawakami (Konan Hospital, Sapporo, Japan) for his constructive comments and E. Yamaguchi (The First Dept of Medicine, School of Medicine, Hokkaido University, Sapporo, Japan) for his valuable advice regarding the flow cytometry technique.

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