



Induction of late airway response was involved in serum antigen-specific immunoglobulin G in rats

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ABSTRACT

The antigen-induced immediate airway response (IAR) has been considered a form of bronchoconstriction mainly provoked by histamine and leukotriene $C_4/D_4/E_4$, which are released by stimulation by antigen-specific IgE. However, the pathophysiological features of the antigen-induced late airway response (LAR) are not yet fully understood. In the present study, sensitized rats were repeatedly exposed to ovalbumin (OVA) to induce IAR and LAR, and the immunological profiles of IAR and LAR were examined. The first antigen inhalation induced only IAR but not LAR. However, the second antigen inhalation 7 days after IAR induced LAR but not IAR. Tumor necrosis factor (TNF)- α level in BALF in LAR was significantly higher than that in IAR, although there were no differences in histamine, leukotriene $C_4/D_4/E_4$, interleukin (IL)-1 β , or IL-13 levels between IAR and LAR. Serum antigen-specific IgE titer was high in both IAR and LAR, but serum antigen-specific IgG, IgG1, and IgG2a titers were dramatically high in LAR but not IAR. There were significant correlations between antigen-specific IgG, IgG1, and IgG2a titers and LAR. Interestingly, LAR could be induced in normal rats by transfer of serum from LAR rats, which exhibited high antigen-specific IgG, IgG1, and IgG2a titers. In conclusion, these findings suggest that repeated antigen inhalation converts IAR to LAR, and that LAR is a reaction triggered by antigen-specific IgG and involving TNF- α . This is the first study to directly suggest the involvement of antigen-specific IgG in the induction of LAR.

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1. Introduction

Asthma has been considered a complex inflammatory disorder of the airways, characterized by various pathophysiological features [1]. Exposure of asthmatics to specific antigen usually results in two phase reactions, an immediate airway response (IAR) and a late airway response (LAR) [2,3]. IAR is caused by quick and strong airway contraction and can be life-threatening for asthmatics, while LAR is chronic condition which continues for several hours and which asthmatics suffer in their daily lives.

The pathophysiological profiles of LAR are different from those of IAR, as indicated by the findings of guinea pig models. It is known that antihistamines suppress IAR but not LAR, while anti-leukotriene $C_4/D_4/E_4$ suppresses both IAR and LAR [4,5]. In addition, β_2 -agonists like salbutamol suppress only IAR but not LAR [6]. These findings indicate that IAR is mediated by histamine and leukotriene $C_4/D_4/E_4$ and is a reaction caused by airway smooth muscle contraction, whereas LAR is mediated by leukotriene $C_4/D_4/E_4$ and is not caused by airway smooth muscle contraction. However, the reason is still unclear why IAR and

LAR have different mechanisms of provoking breathing difficulties despite being induced by the same stimuli, such as specific antigen exposure. The difference between IAR and LAR in pathogenesis is thus still unclear.

Recently, it has been reported that the first antigen exposure to sensitized-animals induced IAR, while further repeated antigen exposure induced LAR in guinea pigs and mice [7–9]. Although the mechanisms of this shift from immediate to late response have yet to be elucidated, these phenomena are useful in elucidating the differences between IAR and LAR. In this study, rats were repeatedly exposed to antigen to induce IAR and LAR, the immunological profiles of IAR and LAR were examined based on the patterns of inflammatory mediators and cells in bronchoalveolar lavage fluid (BALF), and serum antigen-specific immunoglobulin titers were determined.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley (SD) rats were purchased from Charles River Japan, Inc. (CRJ, Yokohama, Japan) and individually housed in plastic cages maintained under specific pathogen-free conditions at a room temperature of 23.0 ± 3.0 °C and air humidity of $55 \pm 15\%$ in a 12-h light/dark cycle environment. The rats were given a standard laboratory

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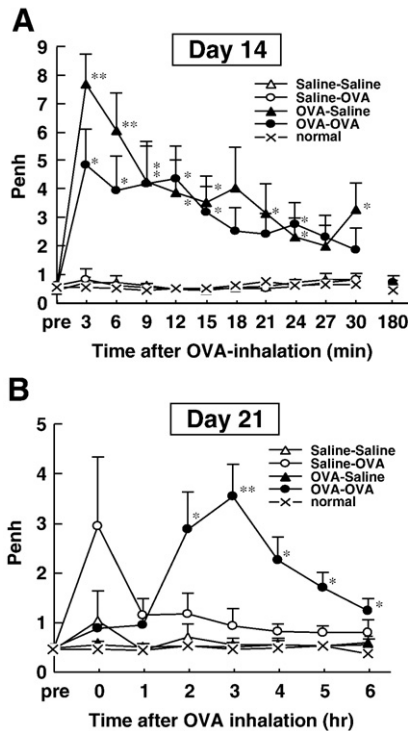


Fig. 1. Time course of changes in airway responses after OVA or saline inhalation on days 14 and 21. Actively sensitized rats inhaled 5% OVA or saline for 5 min using an ultrasonic nebulizer. After inhalation, Penh value was continuously measured by whole-body plethysmography at each time point. Time course of changes in Penh value after inhalation on day 14 (A) and on day 21 (B). The Saline-Saline group inhaled saline on days 14 and 21. The OVA-Saline group inhaled OVA on day 14 and saline on day 21. The Saline-OVA group inhaled saline on day 14 and OVA on day 21. The OVA-OVA group inhaled OVA on day 14 and 21. Values are means $\pm$ S.E. ($n=6$). *, ** $p<0.05$, 0.01 (significantly different from Saline-Saline group).

chow diet (CRF-1, Oriental Yeast, Tokyo, Japan) and water *ad libitum*. All experiments complied with the Guidelines of Animal Experimentation of our laboratories.

2.2. OVA-induced airway responses

SD rats were sensitized on days 0, 1, and 2 with intraperitoneal injection of 10 μ g of OVA (Albumin from chicken egg white grade V, Sigma-Aldrich Co., Tokyo, Japan) and 10 mg of Alum (Imject Alum, PIERCE, Rockford, USA) in saline. On day 14 (first inhalation) and day 21 (second inhalation), rats were challenged with exposure to aerosolized 5% (w/v) OVA in saline for 5 min. Aerosol challenge was performed with groups of up to 4 rats in a closed chamber attached to an ultrasonic nebulizer (NE-U17; Omron Co., Tokyo, Japan). An indicator of airway response (Penh) was measured by barometric plethysmography using whole-body plethysmography (WBP; Buxco, Troy, NY) under the pre-conditioned room temperature and humidity [10,11]. Penh has not yet been frequently measured in rats [12], and potentially existing strain-related differences, such as described in mice [13,14]. However, Kirschvink et al. suggested by comparing Penh with R(L) that Penh can be used as an index of airflow limitation under well-defined experimental condition in SD rats [15]. Rats were placed in the main chamber immediately after OVA exposure, and Penh values were continuously measured for 30 min and averaged for every 3 min period (for day 14) or measured for 8 min and averaged at each time point (for day 21).

In a study of airway responses on days 14 and 21, sensitized rats were randomly divided into 4 groups, i.e. Saline-Saline, Saline-OVA, OVA-Saline, and OVA-OVA groups. In these groups, aerosolized saline or OVA was inhaled on days 14 and 21. For example, the OVA-Saline group inhaled OVA on day 14 and saline on day 21. The normal group

was treated with neither sensitization nor inhalation throughout the experiment.

2.3. Bronchoalveolar lavage fluid (BALF) preparation and analysis

BALF was collected at each time point after OVA challenge. Briefly, rats were deeply anesthetized and exsanguinated from the abdominal aorta. BALF was collected through a bronchial cannula that was connected to a syringe using 8 ml of phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), 2 mM ethylene diamine tetraacetic acid (EDTA), and 10 μ M AA-861 (5-lipoxygenase inhibitor). The number and differential of leukocytes in the BALF were determined using a hematology analyzer (ADVIA 120, Bayer Medical, Tokyo, Japan) that was set in the rat hematology analysis mode. After cell counting, BALF was centrifuged at 1000 $\times$ g at 4 $^{\circ}$ C for 10 min, and the levels of histamine, leukotriene $C_4/D_4/E_4$, IL-13, TNF- α , and IL-1 β in the supernatants were quantified with a histamine enzyme-linked immunosorbent assay (ELISA) kit (Immunotech, Osaka, Japan), leukotriene $C_4/D_4/E_4$ Immunoassay kit (Assay Design, An Arbor, USA), rat IL-13 Immunoassay kit (Biosource, Camarillo, USA), rat TNF-

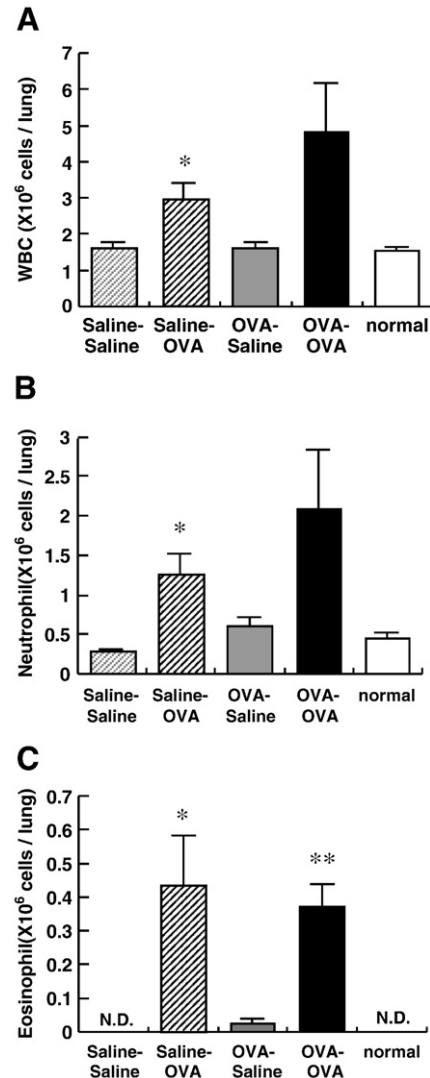


Fig. 2. Numbers of inflammatory cells in BALF on day 22. Twenty-four hours after OVA or saline inhalation on day 21, BALF was collected using 8 ml of PBS containing 0.5% BSA, 2 mM EDTA, and 10 μ M AA-861. The number and differential of leukocytes in BALF were determined using a hematology analyzer. (A) whole blood cell number. (B) Neutrophil number. (C) Eosinophil number. Values are means $\pm$ S.E. ($n=6$). *, ** $p<0.05$, 0.01 (significantly different from Saline-Saline group).

α Immunoassay kit (R&D Systems, Minneapolis, USA), and rat IL-1 β Immunoassay kit (R&D Systems, Minneapolis, USA) according to the manufacturers' protocols.

2.4. Measurement of antigen-specific immunoglobulin titers in sera

Blood samples were collected from the tail vein on day 14 and day 21 just before OVA or saline inhalation. Antigen-specific IgE, IgG, IgG1 and IgG2a in sera were determined using an ELISA technique. For specific IgE measurement, 5 μ g/mL mouse anti-rat IgE (Zymed, South San Francisco, USA) was coated on a 96-well microtiter plate (100 μ L/well) overnight at 4 °C. The plate was washed three times with tap PBS containing 0.5% Tween 20 followed by the addition of 200 μ L/well Block Ace (1:4 diluted in water, Dainippon Seiyaku, Japan). After 1 h incubation at room temperature (RT), the plate was washed, and dilutions of rat serum were added to the wells and incubated for 2 h. After washing, 100 μ L/well peroxidase conjugated streptavidin (DAKO, Tokyo, Japan) was added. After incubation for 1 h at RT, the plate was washed again and 100 μ L/well TMB+Substrate chromogen (DAKO, Tokyo, Japan) was added. The plate was developed at RT for 10 min. Finally, 100 μ L/well of 2N H₂SO₄ was added. Optical densities were read at 450 nm with an ELISA plate reader. For antigen-specific IgG, IgG1, and IgG2a measurements, plates were coated with 1 μ g/mL OVA (100 μ L/well). As a detection antibody, horseradish peroxidase (HRP)-rabbit anti-rat IgG Fc (1:5000 diluted in Block Ace (1:4), BETHYL, Montgomery, USA) was used for specific IgG. Peroxidase-monoclonal mouse anti-rat IgG1 and IgG2a (1:2000 diluted in Block Ace (1:4), Zymed, South San Francisco, USA) were used for specific IgG1 and IgG2a, respectively. For all measurements, dilutions of pre-pooled sera were also measured as reference values to determine the titers in test sera.

2.5. Serum transfer of late airway response (LAR)

Rats were intraperitoneally sensitized with OVA and Alum on days 0, 1, and 2. On day 14, rats were challenged with exposure to an aerosolized 5% OVA for 5 min. On day 21, blood sample (LAR serum) was collected from the abdominal aorta of these rats. Normal serum was collected from normal rats without any OVA treatment. LAR or normal serum was intravenously transferred in the amount of 3 mL into naïve rats. The following day, rats subjected to transfer of LAR serum or normal serum were challenged with exposure to an aerosolized 5% OVA for 5 min, and Penh values were continuously measured after OVA challenge. Penh values were measured and averaged for an 8 min period at each time point.

2.6. Statistical analysis

All results are means $\pm$ standard error (S.E.). For comparisons of two groups, the Aspin-Welch *t*-test (if data were unequal in variance) or Student's *t*-test (if data were equal in variance) was used. For correlation analysis, correlation coefficients and coefficients of determination were calculated by analysis of variance for linear regression, and the Pearson correlation test was used for comparison of paired samples. Findings of *p* < 0.05 were considered significant.

3. Results

3.1. Antigen-induced airway responses on day 14 and day 21

The first antigen inhalation on day 14 produced an immediate increase in Penh value but not a late increase (180 min) in the OVA–Saline group and the OVA–OVA group (Fig. 1A). At the second antigen inhalation on day 21 in the OVA–OVA group, Penh value gradually increased and peaked at 3 h after OVA challenge, while immediate reaction was diminished (Fig. 1B). The third antigen inhalation on day 28 provoked the late airway response but no immediate response, as with the second antigen inhalation (data not shown). On the other hand, the Saline–OVA group on day 21 exhibited a slight immediate increase in Penh value after challenge. Saline inhalation produced no remarkable response on either day 14 or 21.

3.2. Number of inflammatory cells in BALF on day 22

The numbers of inflammatory cells in BALF on day 22 are shown in Fig. 2. In the Saline–OVA group and OVA–OVA group, the numbers of white blood cells (WBC), neutrophils, and eosinophils were significantly increased or tended to increase compared to those in the Saline–Saline group. However, there was no significant difference in numbers of these cells between the Saline–OVA group and OVA–OVA group. In the OVA–Saline group and Saline–Saline group, inflammatory cell numbers exhibited no changes.

3.3. Time course of changes in inflammatory mediators in BALF

The time course of changes in inflammatory cytokines and mediators in BALF on day 21 (LAR) was examined, and compared to that on day 14 (IAR) (shown in Table 1). In the case of LAR, leukotriene C₄/D₄/E₄ levels at 3 h, TNF- α levels at 1 and 6 h, and IL-1 β levels at 0, 1, 3, and 6 h after antigen challenge were significantly increased, while

Table 1
Time course of changes in inflammatory mediators in BALF in IAR or LAR

Mediator	Group		Time after OVA inhalation					
	Day	Inhalation	Pre	0 min	1 h	3 h	6 h	24 h
Histamine (nM)	14	Saline	N.D.	20.7 $\pm$ 6.2	4.4 $\pm$ 3.0	N.D.	N.D.	9.0 $\pm$ 5.6
	(IAR)	OVA		40.5 $\pm$ 23.8	5.6 $\pm$ 5.6	2.0 $\pm$ 2.0	N.D.	N.D.
	21	Saline	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	(LAR)	OVA	3.4 $\pm$ 7.4	N.D.	N.D.	N.D.	N.D.	N.D.
Leukotriene C ₄ /D ₄ /E ₄ (ng/mL)	14	Saline	4.7 $\pm$ 0.5	5.1 $\pm$ 0.5	4.2 $\pm$ 0.4	4.8 $\pm$ 0.9	4.2 $\pm$ 0.7	4.5 $\pm$ 0.5
	(IAR)	OVA		17.1 $\pm$ 4.5	17.4 $\pm$ 6.3	23.9 $\pm$ 7.8	24.1 $\pm$ 9.1	11.5 $\pm$ 2.3 #
	21	Saline	9.0 $\pm$ 5.3	2.7 $\pm$ 1.1	3.1 $\pm$ 0.7	4.7 $\pm$ 1.0	4.9 $\pm$ 2.4	3.4 $\pm$ 1.6
	(LAR)	OVA		7.7 $\pm$ 5.4	5.9 $\pm$ 2.4	8.5 $\pm$ 2.0 #	11.8 $\pm$ 7.3	11.0 $\pm$ 9.0
TNF- α (pg/mL)	14	Saline	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	(IAR)	OVA		N.D.	53.7 $\pm$ 21.2	63.8 $\pm$ 25.3	20.4 $\pm$ 11.0	N.D.
	21	Saline	N.D.	N.D.	N.D.	0.1 $\pm$ 0.1	N.D.	N.D.
	(LAR)	OVA		N.D.	211.0 $\pm$ 54.7 #**	259.9 $\pm$ 172.6 *	47.4 $\pm$ 33.6 ##**	8.2 $\pm$ 12.4
IL-1 β (pg/mL)	14	Saline	N.D.	18.3 $\pm$ 6.4	21.6 $\pm$ 0.3	16.3 $\pm$ 5.5	16.5 $\pm$ 5.5	12.3 $\pm$ 7.1
	(IAR)	OVA		18.0 $\pm$ 4.6	25.6 $\pm$ 0.9 #	51.6 $\pm$ 10.3 #	62.0 $\pm$ 14.7 #	27.9 $\pm$ 3.1
	21	Saline	4.8 $\pm$ 3.1	1.0 $\pm$ 1.5	3.4 $\pm$ 3.2	5.2 $\pm$ 5.0	9.5 $\pm$ 3.2	7.3 $\pm$ 1.8
	(LAR)	OVA		5.1 $\pm$ 2.3 #	19.3 $\pm$ 3.8 ##	32.0 $\pm$ 15.4 #	40.2 $\pm$ 11.1 ##	36.7 $\pm$ 43.8

Values are means $\pm$ S.E. (*n* = 5). N.D.: not detected.

#, ## *p* < 0.05, 0.01 (significantly different from respective saline group).

*, ** *p* < 0.05, 0.01 (significantly different from respective IAR group).

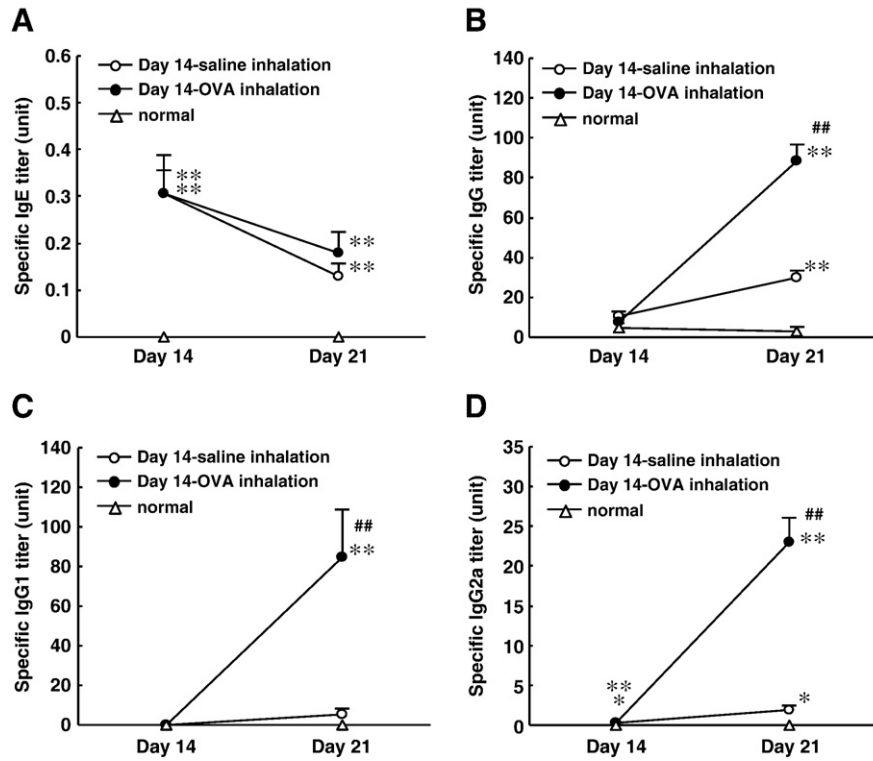


Fig. 3. Changes in serum antigen-specific immunoglobulin titers. Blood sample was collected from the tail vein just before OVA or saline inhalation on days 14 and 21, and OVA-specific IgE, IgG, IgG1, and IgG2a titers in sera were measured by respective ELISA methods. Dilutions of pre-pooled serum were also measured for reference. Open circles represent the day 14-saline inhalation group which inhaled saline on day 14. Black circles represent the day 14-OVA inhalation group which inhaled OVA on day 14. Open triangles represent the normal group which was not sensitized and inhaled nothing. (A) antigen-specific IgE titer. (B) Antigen-specific IgG titer. (C) Antigen-specific IgG1 titer. (D) antigen-specific IgG2a titer. Values are means $\pm$ S.E. ($n=6$ or 12). *, ** $p<0.05$, 0.01 (significantly different from respective normal group). ## $p<0.01$ (significantly different from respective saline (D14) group).

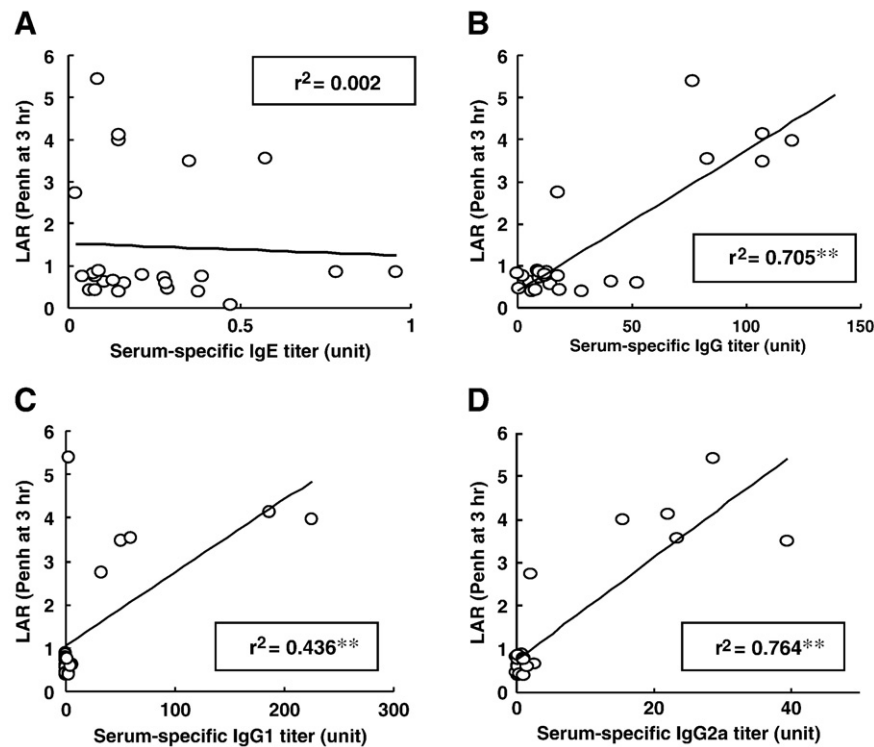


Fig. 4. Correlations between serum antigen-specific immunoglobulin titers and LAR determined by linear regression. Penh value at 3 h after antigen inhalation and serum immunoglobulin titers on days 14 and 21 in same experiment were used for correlation analysis. (A) Relationship between specific IgE and LAR. (B) Relationship between specific IgG and LAR. (C) Relationship between specific IgG1 and LAR. (D) Relationship between specific IgG2a and LAR. r^2 =coefficient of determination. ** $p<0.01$ (significant correlation).

histamine levels were unchanged at each time point. IL-13 levels also were unchanged after antigen challenge (data not shown). The levels of histamine, leukotriene $C_4/D_4/E_4$, and IL-1 β exhibited no differences between IAR and LAR. However, TNF- α levels of LAR at 1, 3, and 6 h were significantly higher than those of IAR.

3.4. Serum antigen-specific immunoglobulin titers on day 14 and day 21

Serum titers of IgE, IgG, IgG1, and IgG2a on days 14 and 21 are shown in Fig. 3A–D, respectively. The titers of specific IgE and IgG2a were increased on day 14 after intraperitoneal sensitization, while those of specific IgG and IgG1 were not. After saline inhalation on day 14, the titer of specific IgE was still significantly high on day 21, and those of specific IgG, IgG1, and IgG2a were slightly increased on day 21. After OVA inhalation on day 14, the titers of specific IgG, IgG1, and IgG2a were dramatically increased on day 21. The titers of specific IgG, IgG1, and IgG2a on day 21 in the day 14-OVA inhalation group were significantly higher than those in the day 14-saline inhalation group.

3.5. Correlations between serum antigen-specific immunoglobulin titers and LAR

To determine the contribution of changes in serum immunoglobulin titers to the induction of LAR, the relationships between the titers of IgE, IgG, IgG1, and IgG2a and LAR (Penh at 3 h after antigen inhalation) on days 14 and 21 were examined by linear regression. There were significant correlations between IgG, IgG1, and IgG2a and LAR (IgG: $r^2=0.705$, IgG1: $r^2=0.436$, IgG2a: $r^2=0.764$), though the correlation between IgE and LAR was not high ($r^2=0.002$) (Fig. 4).

3.6. Serum transfer of late airway response (LAR)

The Penh value in the group with transfer of LAR serum gradually increased and peaked at 1–3 h after antigen challenge, while no immediate increase in Penh value (IAR) was exhibited (Fig. 4). The Penh value in the group with transfer of normal serum was unaffected after OVA challenge.

When IAR serum harvested from rats on day 14 was transferred into naïve rats, only IAR was induced after antigen inhalation (data not shown).

4. Discussion

Specific antigen exposure in asthmatics provokes IAR or LAR under some circumstances. However, the mechanisms of antigen-induced IAR and LAR are still unclear, even though the two responses can be induced by the same stimuli, such as specific antigen exposure. In the present study, we examined the differences in mechanism underlying antigen-induced IAR and LAR in rats. We demonstrated that repeated antigen inhalation converts IAR to LAR, and that serum antigen-specific IgG and TNF- α level in BALF in LAR were significantly higher than those in IAR.

The findings for changes in immunoglobulin titers (Fig. 3), correlation analysis (Fig. 4), and the induction of LAR by serum transfer (Fig. 5) suggest that first antigen inhalation on day 14 increases serum specific IgG titers after IAR, and that this increase in IgG titers is a trigger for induction of LAR at second antigen inhalation. A clinical study suggested that LAR can be induced by antigen inhalation without antigen-specific IgE in some asthmatics, and serum antigen-specific IgG and IgG1 levels in asthmatics with LAR were significantly higher than those in asthmatics without LAR [16,17]. In addition, aged and late-onset asthmatics may produce predominantly IgG1 in response to antigens, and have severe asthma attacks caused by IgG1 rather than IgE. These clinical findings correspond to our findings suggesting that antigen-specific IgG is a trigger of LAR in rats. It has not been determined which subtype of antigen-specific IgG is

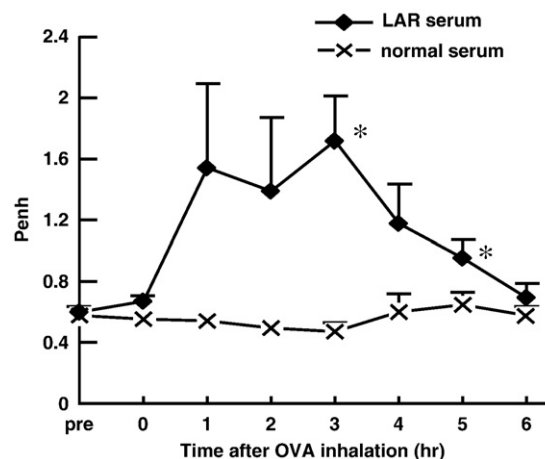


Fig. 5. Time course of changes in airway responses after OVA inhalation in naïve rats with transferral of normal serum or LAR serum. LAR or normal serum was intravenously transferred in the amount of 3 mL into naïve rats. Next day, transferred rats inhaled 5% OVA or saline for 5 min using an ultrasonic nebulizer. After inhalation, Penh value was continuously measured by whole-body plethysmography at each time point. Values are means $\pm$ S.E. ($n=5$). * $p<0.05$ (significantly different from normal serum group).

the most important in inducing LAR, and further evaluation of IgG subtype is needed. However, ours is the first study to directly suggest an association between antigen-specific IgG and antigen-induced LAR.

IAR completely disappeared at second antigen inhalation on day 21 despite the appearance of LAR. In our ex vivo study using the Magnus apparatus, airway isolated from rats with LAR did not exhibit immediate airway contraction by the exposure to antigen, although airway from IAR rats did (data not shown). In a mouse asthmatic model, IAR is decreased by repeated antigen exposure [9], consistent with our findings. However, in a guinea pig asthmatic model, IAR and LAR were commonly provoked together after antigen challenge [18]. It is unclear why IAR disappeared or was diminished in some models of LAR. Some studies have suggested that mast cell responses triggered by high-affinity receptors for IgE may be inhibited by low-affinity receptors for IgG [19], and a significant inverse correlation was found between specific IgG and the immediate airway response [20]. These findings of a negative correlation between IgE-mediated response and IgG-mediated response suggest the possibility that extremely high levels of IgG titers present in LAR suppress the IgE-mediated IAR response in our model. The appearance of IAR may thus depend on the balance of serum specific IgE and IgG titers.

The levels of leukotriene $C_4/D_4/E_4$, IL-1 β , and TNF- α in BALF were increased in LAR, while only TNF- α level in LAR was significantly higher than that in IAR (Table 1). It has been reported that TNF- α and IL-6 secretion by alveolar macrophages contributes to the development of LAR in allergic subjects with asthma [21]. Further, TNF- α is the proximal key cytokine capable of inducing airway hyperresponsiveness (AHR) in mice [22]. Although it is still unclear whether TNF- α in BALF directly induces LAR or not, our findings suggest that TNF- α contributes to antigen-induced LAR in the rat. Further evaluation of the role of TNF- α in LAR is needed.

The role of eosinophils in antigen-induced LAR has been discussed in a study comparing eosinophil number between BALF from subjects or animals exhibiting IAR/LAR and those exhibiting IAR alone [23]. However, it may be necessary to differentiate IAR and LAR to precisely determine the relationships between cell infiltration and each type of airway reaction. In our rat model, since IAR and LAR are independently provoked, it is useful to elucidate the differences between IAR and LAR. We found that the numbers of neutrophils and eosinophils in BALF on day 22 were increased in the group that exhibited LAR on day 21, but also in the group that exhibited only weak IAR on day 21, and that there were no differences in cell number between these two

groups (Fig. 2). In addition, our previous study showed that the numbers of white blood cells (WBC), neutrophils, and eosinophils were equally increased after IAR on day 15 (data not shown). These findings suggest that BALF cell number might not significantly affect airway responses such as IAR and LAR.

In conclusion, our findings suggest that repeated antigen exposure can convert IAR to LAR, and that LAR could be a reaction triggered by antigen-specific IgG, and involving TNF- α . This is the first study directly suggesting the involvement of antigen-specific IgG in the induction of LAR.

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