Repeated Antigen Inhalations Alter Chemical Mediators That Cause Asthmatic Obstruction in Guinea Pigs

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ABSTRACT—The contributions of histamine, cysteiny1 leukotrienes (CysLTs) and thromboxane A₂ (TXA₂) to the asthmatic responses and the magnitudes of blood and lung eosinophilia at acute and chronic stages of our asthmatic model were comparatively determined. Guinea pigs were alternately sensitized/challenged by inhalation with ovalbumin + Al(OH)₃ and ovalbumin, once every 2 weeks. Effects of mepryamine, pranlukast (a CysLT antagonist) and seratrodast (a TXA₂ antagonist) on the early (EAR) and/or the late asthmatic response (LAR) were assessed at the second and fourth antigen challenges. The second challenge caused EAR but not LAR. Although the EAR was decreased at the fourth challenge, a substantial LAR was seen. Both mepryamine and seratrodast inhibited the EAR at the second challenge by approximately 50%. However, at the fourth challenge, these drugs did not inhibit the EAR. The LAR at the fourth challenge was attenuated by pranlukast and seratrodast by 45% and 40%, respectively. Both the blood and lung eosinophilia were modestly and markedly induced 5 h after the second and fourth challenges, respectively. These results strongly suggest that repetition of antigen challenge induces quantitative alterations of chemical mediators participating in the asthmatic responses and a change of the body state under which eosinophils exhibit enhanced migratory activities.

Keywords: Cysteiny1 leukotriene, Eosinophil, Experimental asthma, Histamine, Thromboxane A₂

When patients with allergic asthma are given an inhalation challenge of aerosolized allergen, they develop airway obstruction consisting of early (EAR) and late asthmatic responses (LAR) (1) and airway eosinophilia (2). Various chemical mediators are considered to be involved in these responses. In particular, histamine (3), cysteiny1 leukotrienes (CysLTs) (4) and thromboxane A₂ (TXA₂) (5) induce potent bronchoconstriction, being suggested to contribute to the occurrence of EAR and/or LAR (6–8). In addition, eosinophils infiltrated into the airway are putatively thought to be related to the appearance of LAR (2). It has been reported that the eosinophilia is caused by chemotactic factors such as platelet activating factor (9), LTB₄ (10), interleukin (IL)-5 (11) and eotaxin (12). In addition, CysLTs were recently also observed to induce eosinophil migration into tissues (13).

Chemical mediators involved in the asthmatic responses have been identified mainly by pharmacological analysis of acutely asthmatic animals, which had been sensitized a few times and then challenged once or twice with antigen (14–16). In contrast to a few antigen challenges, repeated allergen challenge has been suggested to aggravate asthma with the appearance of LAR in clinical studies (17). Thus, to develop new anti-asthma drugs and to further understand the mechanisms salient to the clinical aspects of chronic allergic asthma, the chemical mediators inducing the chronic state must be elucidated through the use of an appropriate experimental animal model.

Recently, we have established a repeated antigen inhalation-induced asthmatic model (18): guinea pigs are sensitized and challenged by alternate inhalations of mists of the respective antigen (ovalbumin (OA)) adsorbed on aluminum hydroxide (Al(OH)₃) gels (OA + Al(OH)₃) and OA alone, once every 2 weeks. At the first and the second challenges, EAR is elicited immediately after the OA inhalation challenge, but no late phase airway obstruction is observed. By repeating further sensitzations and challenges, LAR, which occurs 5–7 h after the respective fourth–tenth antigen challenges, also appears (18). In
addition, we have reported that the appearance of the LAR coincides with eosinophilia in the peripheral blood and lung (19).

In the present study, we evaluated effects of a classical antihistaminic, mepyramine; a specific CysLT antagonist, pranlukast (20); and a specific TXA₃ antagonist, seratrodast (16), on EAR and/or LAR at the both the acute (second challenge) and chronic (fourth challenge) stages in our model to investigate the contribution of these mediators to the asthmatic responses. Furthermore, we compared acute and chronic asthma in terms of eosinophilia in the peripheral blood and lung.

MATERIALS AND METHODS

Animals

Male, Hartley guinea pigs, aged 3 weeks and weighing 250–300 g, were purchased from Japan SLC, Hamamatsu. The animals were housed in an air-conditioned room at a temperature of 23 ± 1°C and 60 ± 10% humidity, illuminated from 08:00–20:00 h, fed a standard laboratory diet and given water ad libitum. The first sensitization was started 2 weeks after the purchase. Experimental protocols and animal care method in the experiments were approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

Reagents

Reagents and their sources were as follows: mepyramine maleate (Sigma Chem., St. Louis, MO, USA); pranlukast hemihydrate (supplied by Dr. H. Ohno of Ono Pharm. Co., Osaka); seratrodast (supplied by Dr. Y. Ashida of Takeda Chem. Ind., Osaka); ovalbumin (OA), Triton X-100®, methylcellulose, Tween 20® and H₂O₂ (Wako Pure Chem., Osaka); heparin sodium (Takeda Chem. Ind., Osaka); sodium pentobarbital (Abbott Lab., North Chicago, IL, USA); 3,3',5,5'-tetramethylbenzidine (Dohjindo Lab., Kumamoto); KBr (Merck, Darmstadt, Germany); sucrose and Turk's stain solution (Nacalai Tesque, Kyoto); Diff-Quik® solution (International Reagents, Kobe). The other reagents used were the highest grade of commercial products available.

Mepyramine was dissolved in saline. Pranlukast and seratrodast were suspended in 0.5% methylcellulose.

OA + Al(OH)₃ for sensitization was prepared as previously reported (18, 21). OA solution for challenge was prepared at a concentration of 16 mg/ml.

Sensitization and challenge with antigen

Sensitization and challenge by inhalation of the mists of OA + Al(OH)₃ and OA, respectively, were performed by the previously described method (18) as shown in Fig. 1. In brief, conscious guinea pigs were sensitized by forced inhalation of the OA + Al(OH)₃ mists at a dose of 15 µg OA + 750 µg Al(OH)₃ · animal⁻¹ · time⁻¹, once every 2 weeks for the first 2 times. Then the animal was forced

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**Fig. 1.** Schedule for sensitization and challenge by inhalation with respective mists of ovalbumin (OA) + Al(OH)₃ and OA alone to the guinea pig. *: 15 µg OA + 750 µg Al(OH)₃ · animal⁻¹ · time⁻¹, **: 10 µg OA · animal⁻¹ · time⁻¹. The ordinal number shown in parentheses represents the time of challenge.
to inhale OA mists (10 μg OA·animal⁻¹·time⁻¹) and the OA+Al(OH)₃ mists alternately for challenge and sensitization, respectively, once every 2 weeks until the second (week 8) or the fourth challenge (week 16) (Sensitized—Challenged group). For the negative controls, two groups were prepared: 1) The guinea pigs, which had been sensitized and challenged as described above, were forced to inhale physiological saline mists instead of OA mists at the time corresponding to the second or the fourth challenge (Sensitized—Non-challenged group). 2) The guinea pigs that had been treated with physiological saline mists in place of the antigens once every 2 weeks were treated with physiological saline on the same experimental day as the Sensitized—Non-challenged group (Non-sensitized—Non-challenged group).

In preliminary experiments, more than 60% of the sensitized guinea pigs died immediately (within 20 min) following the first challenge, and the second challenge induced severe anaphylactic symptoms, including labored breathing and cyanosis in the majority of the animals. Therefore, all animals were administered meprynamine prior to the respective first—third challenges except for the experiment for evaluating the effect of meprynamine on asthmatic responses at the second challenge. However, since very few animals exhibited these severe symptoms at the fourth challenge, no meprynamine treatment was performed at the fourth provocation except for in the experiment to determine the effect of meprynamine on asthmatic responses.

Administration of drugs

Pranlukast and seratrodast were administered orally 1 h before the second and the fourth challenges at doses of 30 and 20 mg/kg, respectively. It has been reported that pranlukast (20) and seratrodast (22) at these doses specifically inhibited the respective bronchoconstrictive responses induced by CysLTs and TXA₂, but not those by other agonists in guinea pigs in vivo. Mepyramine (10 mg/kg, i.p.) was administered 30 min before the challenge.

Measurement of the pulmonary function

As described previously (18), airway resistance (Raw) was measured by a two-chambered, double-flow plethysmograph system (Pulmos-I; M.I.P.S., Osaka) according to the method of Pennock et al. (23) before and after the second and the fourth antigen challenges under non-anesthesia.

Blood drawing and counting of leukocytes

Five hours after the second or fourth OA inhalation challenge, peripheral blood was drawn in the presence of 100 U/ml final concentration of heparin from the abdominal vein of each of the Sensitized—Challenged guinea pigs under pentobarbital anesthesia (40 mg/kg, i.p.). Blood samples from the Sensitized—Non-challenged and the Non-sensitized—Non-challenged groups were also drawn 5 h after the saline inhalation in the same manner. The total leukocyte number was determined by staining with Turk's stain. For determining the number of differentiated leukocytes, the blood specimen was centrifuged on the Cell Settling Chamber (Neuro Probe; Cabin John, MD, USA) at 50 × g for 30 s at 4°C after hypotonic treatment, and then the settled leukocytes were stained with Diff-Quik® solution, followed by the microscopic counting of a total of at least 500 cells.

Isolation of the lung and measurement of eosinophil peroxidase (EPO) activity in the lung homogenate

Following the blood drawing, the animals of the respective groups were exsanguinated from the abdominal aorta. After perfusion of the lung with physiological saline via the pulmonary artery, the lung was isolated. The right lower lobe was weighed and used to determine EPO activity. Procedures for the measurement were as follows: the lobe was homogenized (4°C, 12,000 rpm, 20 s × 2) in 10 vol (v/w) of an assay buffer consisting of 0.3 M sucrose in 50 mM acetate buffer (pH 5.4) and then homogenized again (4°C, 8,000 rpm, 20 s × 2) in the presence of 0.2% Triton X-100®. EPO activity in the homogenate was measured as described by Tagari et al. (24) who modified the method of Bozeman et al. (25). A 50-μl aliquot of the 50-fold diluted homogenate was added to 200 μl of the assay buffer with or without 5.3 mM KBr in a polypropylene tube. After adding 100 μl of 3.9 mM 3,3',5,5'-tetrathionylbenzidine containing 2.8 mM H₂O₂ and mixing well, the mixture was incubated for 5 min at 21°C. The peroxidase reaction was terminated by adding 200 μl of 1 N H₂SO₄. After centrifugation (20°C, 1,700× g, 5 min), 200 μl of the supernatant was transferred into a microtiter plate (Maxisorp; Nunc, Roskilde, Denmark), and the optical density at 450 nm was measured. EPO activity was expressed as ΔOD₉₅₄₆0. This method using bromide ion has been reported to be specific for EPO (25).

Statistical analyses

Statistical analysis was performed by one-way analysis of variance. If a significant difference was detected, the individual group difference was determined by Bonferroni's multiple test. A probability value (P) of less than 0.05 was considered to be statistically significant.
RESULTS

Time-course changes of Raw

Time-course changes of Raw following the second and the fourth OA inhalation challenges are shown in Fig. 2. A striking increase (approximately 350% of the pre-challenge value) in Raw corresponding to EAR was observed 10 min after the second challenge. Yet, no increase of Raw 2–10 h after the challenge was seen. At the fourth challenge, the increase in the Raw at the tenth minute was virtually reduced to approximately 170% of the pre-challenged value. However, a late increase of Raw (LAR) was induced at the fifth to tenth hour.

Effect of an antihistaminic drug

Effects of the antihistaminic drug mepyramine on antigen-induced EAR and/or LAR at the second and the fourth antigen challenges are shown in Fig. 3. The drug obviously inhibited the EAR at the second challenge by about 50% (P < 0.05). On the other hand, the EAR at the fourth challenge tended to be minimally suppressed and, in addition, the LAR was hardly influenced by the treatment with mepyramine at the fourth challenge.

Effect of a CysLT antagonist

Figure 4 represents effects of the CysLT antagonist pranlukast on antigen-induced EAR and/or LAR at the second and the fourth antigen challenges. The EAR under the mepyramine treatment at the second challenge was
suppressed by pranlukast by approximately 25%, but the inhibition was not significant. On the other hand, the antagonist significantly inhibited both the EAR and LAR observed at the fourth challenge, each by approximately 45%.

**Effect of a TXA₂ antagonist**

Influences of the TXA₂ antagonist seratrodast on antigen-induced EAR and/or LAR at the second and the fourth challenges are shown in Fig. 5. The EAR at the second challenge was reduced by seratrodast to 55% (P<0.05). In contrast, it did not inhibit EAR at the fourth provocation. However, the drug significantly attenuated the LAR at the fourth challenge by approximately 40%.

**Eosinophilia in the peripheral blood and the lung**

Figures 6 and 7 show the intensity of eosinophilia in the circulatory blood and the lung tissue, respectively, 5 h after the second and the fourth challenges. At the second challenge, eosinophil number in the peripheral blood significantly but minimally increased when compared with those of both the negative controls (the Non-sensitized–Non-challenged and the Sensitized–Non-challenged groups). Considerably different from this, more apparent blood eosinophilia was induced at the fourth challenge. Similar to the behavior of eosinophils in the circulation, the increase of EPO activity in the lung was modest and not significant at the second challenge, but was significant at the fourth one.

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**Fig. 4.** Effect of pranlukast on antigen-induced early (EAR, a and c) and late asthmatic responses (LAR, b and d) at the second (a and b) and the fourth (c and d) antigen inhalation challenges in the sensitized guinea pig. Pranlukast (30 mg/kg) was administered orally 1 h before the challenge. Measurement of airway resistance (Raw) was performed before and at 10 min (EAR, a and c) and 5 h (LAR, b and d) after the challenges. Each column represents the mean±S.E.M. of 13 or 14 animals. □: Non-sensitized–Non-challenged group, ■: Sensitized–Non-challenged group, □: Sensitized–Challenged group (control), □: Sensitized–Challenged–Pranlukast-treated group. *, **: P<0.05, P<0.01.

**Fig. 5.** Effect of seratrodast on antigen-induced early (EAR, a and c) and late asthmatic responses (LAR, b and d) at the second (a and b) and the fourth (c and d) antigen inhalation challenges in the sensitized guinea pig. Seratrodast (20 mg/kg) was administered orally 1 h before the challenge. Measurement of airway resistance (Raw) was performed before and at 10 min (EAR, a and c) and 5 h (LAR, b and d) after the challenge. Each column represents the mean±S.E.M. of 15 animals. □: Non-sensitized–Non-challenged group, ■: Sensitized–Non-challenged group, ■: Sensitized–Challenged group (control), □: Sensitized–Challenged–Seratrodast-treated group. *, **: P<0.05, P<0.01.
**DISCUSSION**

In the present study, contributions of histamine, Cys-LTs and TXA₂, all thought to be important chemical mediators in asthma, to the increase of Raw in our recently developed asthmatic model, were assessed. Consistent with our previous report (18), the second antigen challenge caused EAR but not LAR. At the fourth challenge, EAR intensity was lessened, while LAR appeared. Table 1 summarizes the effects of some highly selective antagonists of histamine, Cys-LTs and TXA₂ on these EAR and LAR. Histamine was one of the predominant mediators responsible for the EAR at the second challenge, and TXA₂ was also shown to be significantly involved. However, participation of histamine and TXA₂ in EAR at the fourth challenge was greatly reduced. The participation of Cys-LTs in production of the EAR was higher at the fourth challenge than the second one. The LAR at the fourth challenge was not mediated by histamine, but partly induced by both Cys-LTs and TXA₂. In contrast to the acute stage, marked eosinophilia in the peripheral blood and lung tissue was elicited by the fourth challenge. These results indicate that the alteration of chemical mediators participating in the asthmatic responses and pronounced eosinophilia are induced by repeated antigen exposures.

Guinea pigs have been frequently used as an acute asthmatic model because their respiratory system exhibits some close similarities to that of humans, including the respiratory organ being mainly attacked in anaphylaxis, histology and responsiveness to chemical mediators (26, 27). Still, different from humans, most researchers have regarded guinea pig airway tissue to be an extraordinarily high responder to histamine released in anaphylaxis. Therefore, antihistaminics have been used in almost all studies on allergic bronchial asthma in guinea pigs to prevent death from anaphylactic shock. In contrast, no anaphylactic deaths were induced at the fourth challenge of our model without the treatment of mepyramine. Moreover, although the present effectiveness of seratrodast on the EAR at the second challenge was similar to that of another model (16), which was prepared acutely for 2 weeks, the EAR induced by the fourth challenge was not influenced by the TXA₂ antagonist. These observations strongly suggest that the mechanisms causing the EAR at the fourth challenge are quite different from those of the second challenge of our model and other non-chronic asthmatic models (14, 16).
We are currently performing investigations on the mechanisms responsible for deterioration of EAR by repetitive antigen exposure in relation to the decreased involvement of histamine in EAR. Results obtained from the preliminary study are as follows: 1) In agreement with results of Andrew et al. (28), alterations of in vitro contractile responses of the trachea, bronchus and lung parenchyma to histamine are not likely to be induced by the repeated antigen challenge. 2) No apparent difference in the in vitro releasability of histamine from lung fragments induced by antigen is seen between the acute and the chronic stages (S. Kohno et al., unpublished data). Therefore, changes of other factors must contribute to the deterioration. For example, activities of histamine-degrading enzymes such as histamine N-methyltransferase might be increased by the chronic antigen provocation. On the other hand, the serum levels of anaphylactic antibodies are apparently unrelated to the deterioration in EAR because our previous report demonstrated that antigen-specific γ1 and IgE antibodies are in fact increasing during the second to fourth challenge (18).

Although mast cells are considered to be the cellular source of histamine in EAR (29), the source of TXA₂ is unclear. Both in vitro and in vivo, guinea pig lung was shown to generate TXA₂ when stimulated with CysLTs (30). However, since the CysLT antagonist did not significantly inhibit EAR at the second challenge, this cyclooxygenase product may be generated from unidentified cells directly by an antigen-antibody reaction or indirectly through some mediators other than CysLTs. Bachelet et al. (31) have reported that alveolar macrophages obtained from sensitized guinea pigs can produce TXA₂ in response to antigen.

The clinical effect of antihistaminic drugs on the late bronchoconstrictive response is controversial (32). The majority of antihistaminic drugs were found to be ineffective against LAR (33, 34). In this study, mepyramine did not inhibit the LAR occurring at the fourth challenge. In addition, participation of histamine in the EAR decreased as antigen challenges were repeated. These observations further suggest that antihistaminic drugs may not be therapeutically ineffective for chronic asthma.

We have proposed the possibility that repetitive exposures to OA + Al(OH)₃ may induce some alterations in the immune system because LAR is not induced by repeated inhalations of OA alone (18). The mechanism for the occurrence of the LAR by repeated antigen inhalations is still not clearly understood. Nevertheless, the LAR observed in the present study was partly inhibited by pranlukast, suggesting that CysLTs play a role in LAR. In addition, when the CysLT antagonist was administered 3 h after the fourth provocation, inhibition of the LAR similar to the present result was seen (T. Nabe et al., submitted manuscript). The cellular source of CysLTs in the LAR is not clear. It has been reported that IL-5 potentiates CysLT production by human eosinophils (35). In addition to this, Coéffier et al. (36) have suggested that IL-5 endows eosinophils with enhanced migratory activities in guinea pigs. From these observations, eosinophils are likely to be primed by some cytokines such as IL-5 at the chronic stage, so that they can easily infiltrate into the lung and produce CysLTs by themselves.

TXA₂ can also mediate the LAR because this response was diminished by seratrodast. Considering the fact that guinea pig eosinophils are capable of producing a large amount of TXA₂ in response to various biologically active substances (37, 38), the TXA₂ production in the LAR may be attributable to migrated eosinophils.

In conclusion, repeated antigen inhalation alters chemical mediators participating in the early and late asthmatic responses in guinea pigs. In addition, the recruitment of eosinophils into the peripheral blood and lung tissue is potentiated by the chronic exposures to antigen.

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