

AN AIRWAY HYPERRESPONSIVENESS MODEL IN RAT ALLERGIC ASTHMA

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We attempted to obtain a new airway hyperresponsiveness model using DNP-*Ascaris* extract (DNP-Asc)-induced rat allergic asthma. Male Wistar rats were actively sensitized with DNP-Asc, and challenged in a non-anesthetized state by inhalation of the antigen for 10 min in a chamber. One, 6 and 24 hr after DNP-Asc challenge, the responsiveness of the airway smooth muscles to inhaled acetylcholine (ACh) was determined using a modified Konzett-Rössler method under anesthesia. Twenty four hr after the challenge, a significant and marked airway hyperresponsiveness was seen. The increase in airway responsiveness was significantly inhibited by pretreatments with a leukotriene antagonist, ONO-1078, and a thromboxane synthetase inhibitor, ozagrel, and tended to be inhibited by a PAF antagonist, CV-3988. The hyperresponsiveness induced by DNP-Asc challenge was accompanied by airway inflammation determined by dye exudation. From the above results, it is indicated that a model of airway hyperresponsiveness was established in rats with allergic asthma, and that the chemical mediators involved in the response might be leukotrienes, thromboxane A_2 and PAF.

Key words: allergic asthma — chemical mediators — DNP-*Ascaris* — hyperresponsiveness — rat

INTRODUCTION

Airway hyperresponsiveness, as defined by an increased contractile response of airway smooth muscle to diverse stimuli, is an essential feature of asthma¹⁾. The precise mechanisms underlying hyperresponsiveness in asthma, however, remain unknown. It is clear that if treatment of airway hyperresponsiveness were feasible, great progress in asthma therapy might be accomplished.

So far, several animal models of airway hyperresponsiveness have been made. The atmospheric pollutant, ozone, causes a transient increase in non-specific hyperresponsiveness in the dog²⁾³⁾, guinea pig⁴⁾ and men⁵⁾. Toluene diisocyanate also causes airway hyperresponsiveness in guinea pigs⁶⁾⁷⁾ and man⁸⁾. Platelet-activating factor (PAF), which was first recognized in the circulation of sensitized rabbits following acute anaphylaxis,

induces potent and prolonged airway hyperresponsiveness in guinea pigs⁹⁾, rhesus monkeys¹⁰⁾, dogs¹¹⁾ and man¹²⁾.

On the other hand, it is also known that inhalation of allergen results in an increase in bronchial responsiveness in atopic asthmatics^{13)–15)} and in laboratory animals. In ragweed-sensitized dogs and rabbits, the development of bronchial hyperresponsiveness was observed after allergen challenge¹⁶⁾¹⁷⁾. Such allergy-induced airway hyperresponsiveness has also been reported in ovalbumin-provoked guinea pigs¹⁸⁾¹⁹⁾, and *Ascaris*-provoked dogs²⁰⁾, rhesus monkeys²¹⁾²²⁾ and sheep²³⁾.

Recently, rats have been used for experimental asthma models to screen antiallergic drugs. Rat allergic asthma model has some similarities to human bronchial asthma in the following points. The antibody species responsible for rat allergic asthma model is mainly IgE, instead of IgG which is mainly involved in guinea pig allergic asthma. Antihistamines have their limitation in controlling asthma in rats and humans, while in guinea pigs. Disodium cromoglycate is effective in rat and human asthma, but not in guinea pig asthma. So far, few detailed investigations of airway hyperre-

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Abbreviations: ACh acetylcholine; DNP-Asc dinitrophenylated *Ascaris suum* extract; EB Evans blue; LAR late asthmatic response; PAF platelet-activating factor; VO ventilation overflow

sponsiveness in rats have been carried out.

In the present study, the development of an animal model to study airway hyperresponsiveness was attempted by using rat allergic bronchial asthma induced by DNP-*Ascaris* (DNP-Asc). Moreover, the chemical mediators involved in airway hyperresponsiveness and in the accompanying airway vascular exudation were investigated using mediator antagonists.

MATERIALS AND METHODS

1. Sensitization and antigen challenge

Male Wistar rats (220 to 320 g) were purchased from Charles River Japan, Inc. The animals were immunized with DNP-Asc (2 mg protein, s.c.) together with killed *Bordetella pertussis* (2×10^{10} , i.m.) as an adjuvant and were boosted by DNP-Asc (0.5 mg protein, s.c.) 5 days later according to the method of Tada and Okumura²⁴. Eight days after the first immunization, they were challenged in a non-anesthetized state by inhalation of the antigen (3 mg or 6 mg protein) for 10 min in a chamber (30 × 25 × 15 cm). In the control group, the rats inhaled saline instead of the antigen at the challenge point. The antibody titer against DNP-Asc determining from 48 hr PCA was in a range of 8 to 256 averaging 40.

2. Bronchial responsiveness to aerosolized acetylcholine (ACh)

One, 6 and 24 hr after the DNP-Asc challenge, the animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and immobilized with pancuronium bromide (1 mg/kg, i.v.). They were ventilated artificially through a tracheal cannula at a frequency of 70 breaths/min. The preparation used in the present study was described previously²⁵. In anesthetized rats, the bronchomotor tone was measured by a modification of the Konzett-Rössler method²⁶. The lung was inflated with a fixed volume of air (tidal volume 3–6 ml) at room temperature and humidity under a constant pressure of 5 cmH₂O, and continuous recordings of the changing ventilation overflow (VO) were made with a pneumotachograph (MFP-1T, Nihon Kohde, Tokyo, Japan) for an index of change in airway resistance. The basal VO was set up at 1

ml at the beginning of experiment. Systemic arterial blood pressure was monitored with a pressure transducer (MPU-0.5, Nihon Kohden, Tokyo, Japan) from a cannula inserted into the left carotid artery, and heart rate was measured with a tachometer (RT-5, Nihon Kohden, Tokyo, Japan) using systolic blood pressure as the trigger. All the above parameters were recorded on a polygraph (RM-85, Nihon Kohden, Tokyo, Japan).

To determine the change in airway hyperresponsiveness, the bronchoconstriction induced by ACh was used as the index. ACh solutions (0.003–0.3%, 5 concentrations) were aerosolized with an ultrasonic nebulizer (TUR-3000, Nihon Kohden, Tokyo, Japan) with a specially devised plastic cylindrical chamber²⁵), and the animals inhaled them from the lowest concentration. When the increase in VO induced by a given ACh concentration recovered to the base-line, the next concentration of ACh was inhaled.

To investigate the chemical mediators involved in airway hyperresponsiveness, the following drugs were administered before DNP-Asc challenge: chlorpheniramine (H₁ blocker; 1 mg/kg, i.p. 30 min before), indomethacin (cyclooxygenase inhibitor; 5 mg/kg, i.p. 1 hr before), ONO-1078 (leukotrienes C₄/D₄ antagonist; 10 mg/kg, i.p. 30 min before), CV-3988 (PAF antagonist; 3 and 10 mg/kg, i.v. 5 min before), and ozagrel (thromboxane A₂ synthetase inhibitor; 100 and 300 mg/kg, p.o. 30 min before the challenge).

3. Determination of airway microvascular leakage

Twenty-four hr after antigen challenge, rats were anesthetized with urethane (2 g/kg, i.p.). Airway vascular permeability was quantified by the extravasation of Evans blue dye (EB) using a modification of the method of Evans et al.²⁷), which correlates well with the extravasation of radiolabeled albumin²⁸. EB (30 mg/ml in saline, filtered with paper filter), 30 mg/kg, and heparin (600 U/kg) were administered by i.v. injection. Twenty min later, the animals were exsanguinated from the abdominal aorta, the thorax was opened and a blunt-ended 13-gauge needle was passed through the right ventricle into the pulmonary artery. The pulmonary veins were incised to allow the outflow of perfusate, and the airways and lungs were perfused with 20–30 ml of 1%

paraformaldehyde in citrate-buffered saline (pH 3.5) to remove the intravascular dye and to fix the tissues. After that, the main bronchus was removed and the wet weight of the tissue was measured.

EB was extracted by incubating the tissue in 1 ml of 100% formamide at 60°C for 24 hr, and its concentration was determined by light absorbance at 620 nm (U-2000 spectrophotometer, Hitachi, Japan) and by interpolation on a standard curve of EB concentrations (3.125–50 µg/ml). The EB content of each sample was expressed as nanogram per milligram wet weight of tissue.

4. Drugs

The drugs used were: pentobarbital sodium (Tokyo Kasei, Tokyo, Japan), chlorpheniramine maleate (Tokyo Kasei, Tokyo, Japan), indomethacin (Sigma, St. Louis, MO), ONO-1078 (4-oxo-8-p-(4-phenylbutoxy) benzoylamino-2-(tetrazol-5-yl)-4H-1-benzopyran hemihydrate, Ono Pharmaceutical Co., Osaka, Japan), ozagrel (OKY-046; (E)-3-4-(1-imidazol-1-yl-methyl) phenyl-2-proenoic acid hydrochloride monohydrate, Kissei Pharmaceutical Co., Matsumoto, Japan), CV-3988 ((RS)-2-methoxy-3-(octadecylcarbamoyloxy) propyl-2-(3-thiazolo) ethyl phosphate, Takeda Pharmaceutical Co., Osaka, Japan), acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo, Japan), pancuronium bromide (Sankyo Co., Tokyo, Japan), Evans blue (Merck Co., NJ, USA) and heparin sodium (Kodama Co., Tokyo, Japan).

5. Statistics

All values were expressed as the mean with S.E.M. Statistical significance of difference was determined by Student's *t*-test.

RESULTS

1. Bronchial responsiveness to acetylcholine

When conscious sensitized rats were challenged in an acryl chamber with DNP-Asc (3 or 6 mg protein), the following signs were observed: nose scratching, dyspnea, abdominal posture and blepharoptosis. The dose-response curves of change in ventilation overflow (VO) induced by ACh aerosols (0.003%–0.3%) 1 hr after DNP-Asc anti-

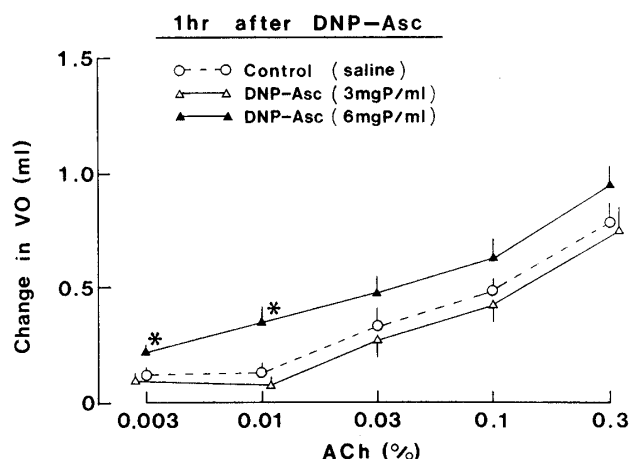


Fig. 1. Dose-response curves of changes in ventilation overflow (VO) induced by ACh aerosol 1 hr after challenge with DNP-Asc in anesthetized rats. Each point represents the mean with S.E.M. from 6 experiments. *: $p < 0.05$ vs. control.

gen challenge are shown in Fig. 1. The VO changes caused by inhalations of ACh were not significantly altered by the challenge with DNP-Asc at a concentration of 3 mg protein/ml. However, DNP-Asc at a concentration of 6 mg protein/ml caused a significant increase or a tendency to increase in the ACh-induced response. Airway responsiveness to inhaled ACh 6 hr after antigen challenge (6 mg protein/ml) also tended to increase (Fig. 2).

Twenty four hr after DNP-Asc challenge, airway responsiveness to inhaled ACh was markedly and significantly increased at either concentration of DNP-Asc (Fig. 3). The VO response to 0.03% and 0.3% ACh was enhanced by 239% and 197%, 24 hr after DNP-Asc challenge at a concentration of 6 mg protein/ml, respectively.

On the other hand, airway responsiveness to i.v. administration of ACh (10–1000 µg/kg, 5 doses) was not significantly different from that of the control group, even 24 hr after DNP-Asc challenge (Fig. 4).

The effects of several antagonists on airway hyperresponsiveness to ACh that were observed 24 hr after DNP-Asc (6 mg protein/ml) challenge, are shown in Fig. 5. The enhanced increase in VO (4.72 ± 0.80 vs. 2.28 ± 0.35 ml min as control) after DNP-Asc challenge was significantly inhibited by pretreatments with ONO-1078 (10 mg/kg, i.p.)

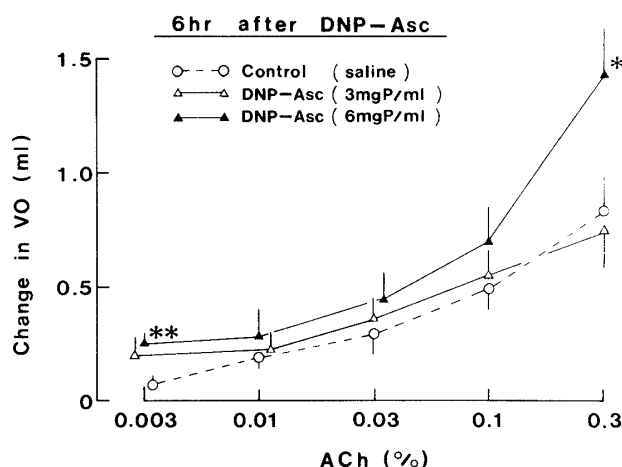


Fig. 2. Dose-response curves of changes in ventilation overflow (VO) induced by ACh aerosol 6 hr after challenge with DNP-Ascaris antigen (DNP-Asc) aerosol in anesthetized rats. Each point represents the mean with S.E.M. from 7 (control), 5 (DNP-Asc; 3 mg protein/ml), and 6 (DNP-Asc; 6 mg protein/ml) experiments. *: $p < 0.05$ and **: $p < 0.01$ vs. control.

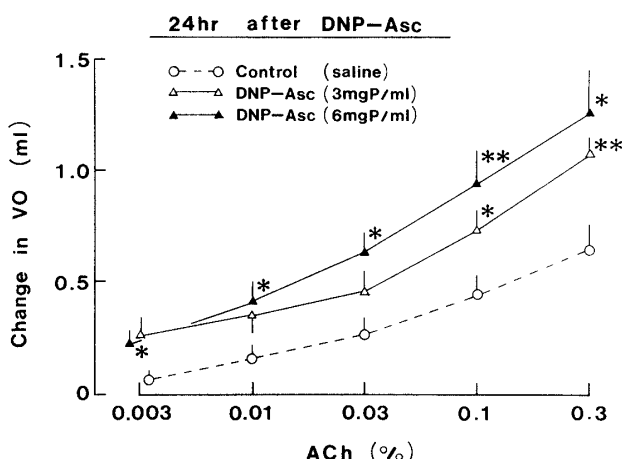


Fig. 3. Dose-responses curves of changes in ventilation overflow (VO) induced by ACh aerosol 24 hr after challenge with DNP-Ascaris antigen (DNP-Asc) aerosol in anesthetized rats. Each point represents the mean with S.E.M. from 7 (control), 8 (DNP-Asc; 3 mg protein/ml), and 7 (DNP-Asc; 6 mg protein/ml) experiments. *: $p < 0.05$ and **: $p < 0.01$ vs. control.

(2.34 ± 0.32 ml min, $p < 0.05$) and ozagrel (300 mg/kg, p.o.) (2.67 ± 0.21 ml min, $p < 0.05$). pretreatments with CV-3988 (3 and 10 mg/kg, i.v.) indomethacin (5 mg/kg, i.p.), and chlorpheniramine (1 mg/kg, i.p.) tended to inhibit the enhanced bronchomotor response. Pretreatment with a combination of ozagrel (300 mg/kg, p.o.) and CV-3988

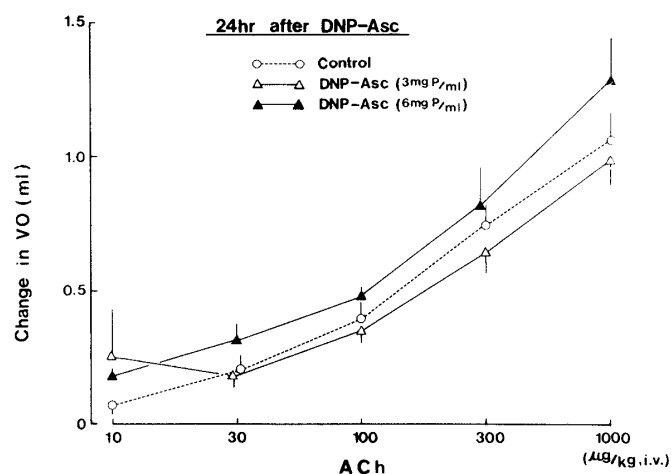


Fig. 4. Dose-response curves of changes in ventilation overflow (VO) induced by i.v. administration of ACh, 24 hr after challenge with DNP-Ascaris antigen (DNP-Asc) aerosol in anesthetized rats. Each point represents the mean with S.E.M. from 7 (control), 5 (DNP-asc; 3 mg protein/ml), and 6 (DNP-Asc; 6 mg protein/ml) experiments.

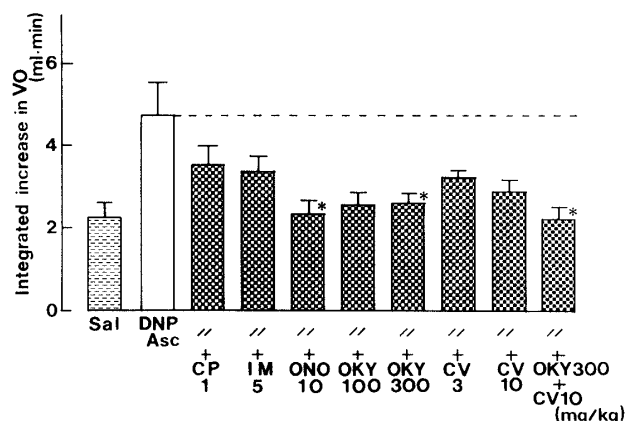


Fig. 5. Effects of chlorpheniramine (CP), indomethacin (IM), ONO-1078 (ONO), ozagrel (OKY), CV-3988 (CV) and ozagrel plus CV on the integrated increases in VO induced by ACh aerosol 24 hr after challenge with DNP-Ascaris antigen (DNP-asc) aerosol in anesthetized rats. Sal.: saline inhalation instead of DNP-Asc. The doses (mg/kg) of drugs used are shown in the figure. The routes of administration are as follows: CP, i.p.; IM, i.p.; ONO, i.p.; OKY, p.o.; CV, i.v. Each column represents the mean with S.E.M. from 6 to 8 animals. *: $p < 0.05$ and **: $p < 0.01$ vs. DNP-Asc alone group.

(10 mg/kg, i.v.) stopped the enhancement.

2. Change in airway microvascular leakage after antigen challenge

Vascular permeability in the bronchus was

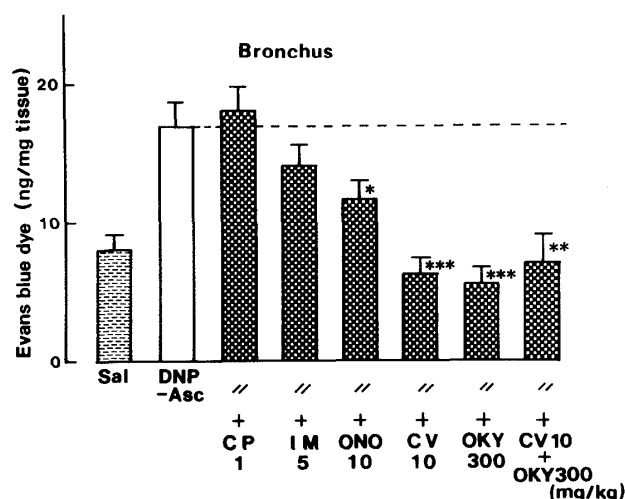


Fig. 6. Effects of several antagonists on the extravasation of Evans blue dye in the rat bronchi 24 hr after DNP-*Ascaris* antigen challenge. ***: $p < 0.001$ vs. DNP-Asc alone group. Other explanations are as in Fig. 5.

markedly and significantly ($p < 0.001$) increased 24 hr after DNP-Asc challenge (Fig. 6), although it was not 1 and 6 hr after DNP-Asc challenge (data not shown). The increase in vascular permeability was inhibited by pretreatments with ONO-1078, CV-3988 and oxagrel.

DISCUSSION

Recently, rats have been used as experimental models of allergic asthma to screen antiallergic drugs. Evans et al.²⁹⁾ suggested that airway hyperresponsiveness occurs after ozone challenge in rats. Sapienza et al.³⁰⁾ reported that responsiveness to methacholine increased after repeated ovalbumin challenge in Brown Norway rats. They proposed that there may be a relationship between the quantity of airway smooth muscle in the large airways and change in responsiveness to methacholine. However, there has been no detailed investigation of airway hyperresponsiveness with reference to airway inflammation and the chemical mediators involved using rat models of allergic asthma.

In the present study, it was found that airway responsiveness to ACh was markedly increased 24 hr after DNP-Asc challenge in sensitized rats. Such marked airway hyperresponsiveness was

not seen 1 hr after DNP-Asc challenge. It is thus suggested that the mediators responsible for immediate asthmatic response (IAR) do not directly cause airway hyperresponsiveness. In humans, a late asthmatic response (LAR) reportedly begins to occur about 4 or 6 hr after IAR induced by allergen inhalation. In the present study, a tendency for airway responsiveness to increase was observed 6 hr after DNP-Asc challenge, which corresponds with the results observed after allergen challenge in sheep³²⁾ and humans¹³⁾. In sheep, airway hyperresponsiveness to inhalation of ACh occurs after *Ascaris* antigen challenge^{32/33)}, probably through the mediation of neutrophils and eosinophils infiltrated in airway tissues³⁴⁾. There is, therefore, a possibility that the same kind of mechanisms are responsible for airway hyperresponsiveness after antigen challenge in rats. Contrary to what happens in ACh inhalation, airway responsiveness after i.v. administration of ACh was not changed. James and coworkers³⁵⁾ found that after inhalation of cigarette smoke in guinea pigs, airway hyperresponsiveness was induced by ACh inhalation, but not by i.v. administration of ACh, suggesting the importance of airway epithelium in airway hyperresponsiveness caused by ACh inhalation. Their finding is likely to have some relevance to the interpretation of our results.

Airway hyperresponsiveness observed at 24 hr after DNP-Asc challenge was significantly inhibited by pretreatments with ONO-1078 (leukotriene C₄/D₄ antagonist) and oxagrel (thromboxane A₂ synthetase inhibitor), and tended to be inhibited by CV-3988 (PAF receptor antagonist), which suggests that the chemical mediators involved in this airway hyperresponsiveness might be leukotrienes, thromboxane A₂ and PAF. The doses of antagonists used were the doses that specifically antagonize the respective agonists^{36)–39)}. In other hyperresponsiveness models, airway hyperresponsiveness was inhibited by a leukotriene antagonist FPL-57213³²⁾ and a PAF antagonist YM-461 in sheep⁴⁰⁾, and by oxagrel in dogs⁴²⁾. The group of Nadel et al. stated that airway hyperresponsiveness results from an enhanced generation of thromboxane A₂ in neutrophils which were stimulated by leukotriene B₄, PAF or antigen^{11/41/42)}. It has been reported that a thromboxane A₂ mimetic, STA₂, caused airway hyperresponsiveness in

dogs⁴³⁾ and guinea pigs⁴⁴⁾. Plasma thromboxane B₂ was increased in both the immediate and late asthmatic responses in asthmatic patients induced by allergens⁴⁵⁾. Also, a thromboxane A₂ receptor antagonist, AA-2411, inhibited airway hyperresponsiveness in patients with asthma⁴⁶⁾. Ozagrel is now commercially available for a therapy drug of airway hyperresponsiveness in our country.

PAF has been reported to induce bronchoconstriction mainly through the mediation of leukotrienes and thromboxane A₂ in guinea pig lungs⁴⁷⁾. PAF also increases leukotrienes⁴⁸⁾⁴⁹⁾ and thromboxane A₂⁴⁶⁾ production in rat lungs. On the other hand, leukotrienes, in turn, increase PAF production in human endothelial cells⁵⁰⁾. Moreover, the generation of prostaglandin E₂, prostacyclin and thromboxane A₂ is stimulated by leukotrienes in activated rat macrophages⁵¹⁾. These findings suggest that the chemical mediators released in bronchial asthma might work in an extremely complex, interrelated manner. In the present study, the inhibitions of airway hyperresponsiveness by ONO-1078, ozagrel and CV-3988 were not of additive type, but were reminiscent of such complicated interactions among these chemical mediators.

An increase in vascular permeability was also seen in the bronchus 24 hr after antigen challenge. The increased response was inhibited by pretreatment with ONO-1078, CV-3988 and ozagrel, as in the case of airway hyperresponsiveness. Therefore, it seems that there may be a relationship between airway hyperresponsiveness and airway inflammation in the present rat model, although it seemed that thromboxane A₂ and PAF might be more potent in increasing dye exudation than leukotrienes, while leukotrienes, thromboxane A₂ and PAF were almost the same potency in inducing airway hyperresponsiveness.

In conclusion, DNP-Asc antigen challenge in sensitized rats reproducibly causes airway hyperresponsiveness. This IgE-mediated²⁴⁾ airway hyperresponsiveness model may be useful for studying the mechanisms of airway hyperresponsiveness and screening antiasthmatic drugs from the point of view of airway hyperresponsiveness.

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ラットアレルギー性喘息の気道過敏性モデル

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DNP-Ascaris 抗原で誘発したラットアレルギー性喘息を用いて新しい気道過敏性モデルの作製を試みた。雄性 Wistar ラットを DNP-Ascaris 抗原で能動感作し、無麻酔下で10分間抗原吸入によりチャレンジした。チャレンジの1, 6 および24時間後に麻酔下で吸入 acetylcholine に対する気道平滑筋の反応性を Konzett-Rössler 法変法で測定した。チャレンジの24時間後に有意で著明な気道反応性の亢進が観察された。この気道過敏性は leukotriene 拮抗薬 ONO-1078 および thromboxane 合成酵素阻害薬 oza-grel の前処置で抑制され、PAF 拮抗薬 CV-3988 によって抑制傾向が認められた。この DNP-Ascaris 誘発喘息によって気道過敏性が発現する時点では気道の血管透過性亢進も観察された。以上の成績から、新しい気道過敏性モデルがアレルギー性喘息ラットで作製でき、この気道過敏性発現には leukotrienes, thromboxane A_2 および PAF が関与していることが示唆された。