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Lipopolysaccharide exposure makes allergic airway inflammation and hyper-responsiveness less responsive to dexamethasone and inhibition of iNOS

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Clinical and Experimental Allergy

Summary

Allergic airway disease can be refractory to anti-inflammatory treatment, whose cause is unclarified. Therefore, in the present experiment, we have tested the hypothesis that co-exposure to lipopolysaccharide (Lps) and allergen results in glucocorticoid-resistant eosinophil airway inflammation and hyper-responsiveness (AHR). Ovalbumin (Ova)-sensitized BALB/c mice were primed with 10 µg intranasal Lps 24 h before the start of Ova challenges (20 min on 3 consecutive days). Dexamethasone (5 mg/kg/day) was given on the last 2 days of Ova challenges. AHR, cellular build-up, cytokine and nitrite concentrations of bronchoalveolar lavage fluid (BALF) and lung histology were examined. To assess the role of iNOS-derived NO in airway responsiveness, mice were treated with a selective inhibitor of this enzyme (1400W) 2 h before AHR measurements. More severe eosinophil inflammation and higher nitrite formation were found in Lps-primed than in non-primed allergized mice. After Lps priming, AHR and concentrations of T-helper type 2 cytokines in BALF were decreased, but still remained significantly higher than in controls. Eosinophil inflammation was partially, while nitrite production and AHR were observed to be largely dexamethasone resistant in Lps-primed allergized animals. 1400W effectively and rapidly diminished the AHR in Ova-sensitized and challenged mice, but failed to affect it after Lps priming plus allergization. In conclusion, Lps inhalation may exaggerate eosinophil inflammation and reduce responsiveness to anti-inflammatory treatment in allergic airway disease.

Keywords asthma, eosinophil airway inflammation, hyper-responsiveness, lipopolysaccharide, nitric oxide, ovalbumin, steroid, 1400W

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Introduction

Lipopolysaccharide (Lps, bacterial endotoxin) is a ubiquitous environmental pollutant present in air [1], household [2] and organic dust [3]. Lps inhalation has been considered to contribute to both the induction of airway allergy and the worsening of asthma symptoms. According to Eisenbarth et al. [4], the co-exposure to low concentrations of Lps was indispensable for the induction of airway allergy in mice, and according to Strohmeier et al. [5], without Lps binding protein (necessary for Lps recognition) allergized mice did not develop airway hyper-responsiveness and showed no increase of airway peroxynitrite, which is a highly pro-inflammatory agent [6].

Lps could exacerbate airway inflammation in individuals with established allergic asthma [2], who have also proven to be more sensitive to the bronchoconstrictive

effects of Lps [7]. In addition, inhalation of Lps decreased to 1/5th the PD20 of allergens in asthmatic patients [8].

Purely allergic mechanisms of inflammation respond quite well to glucocorticoids [9], but among patients with bronchial asthma, there is a great variability of steroid responsiveness [10], whose cause remains mostly unknown. Of note, inflammation and bronchoconstriction induced by Lps may be hardly treatable by glucocorticoids in humans [11, 12], as well as mice [13]. During recent years, several experimental studies on airway effects of Lps plus allergen exposure-induced inflammation have been published [4, 5, 14–19], but none of them have investigated how Lps co-administration modifies treatability of allergic airway inflammation and hyper-responsiveness. Therefore, the present experiment was aimed at (1) establishment and characterization of a mouse model of airway eosinophil inflammation and airway

hyper-responsiveness (AHR) as induced by priming with Lps and subsequent challenges by inhaled ovalbumine (Ova) in previously sensitized mice and (2) comparison of the effects of dexamethasone (Dex) between mice with Lps+allergy and others with pure allergic airway disease (without Lps priming).

Bronchial NO is a major mediator of both allergic and non-allergic airway inflammation [20–22] and inducible NO synthase (iNOS) inhibitors are also potential anti-asthma agents [23]. Therefore, the concentration of nitrite (NO_2^-) – the primary metabolite of NO – was measured in bronchoalveolar lavage (BAL) fluid and the effect of 1400W, a new, highly selective inhibitor of iNOS [24], was tested.

Methods

Animals

Six- to eight-week-old, female, specified pathogen-free BALB/c mice were obtained from Charles River Laboratories (Isaszeg, Hungary). Mice were barrier-housed in high-efficiency particulate-filtered air-ventilated, climate-controlled ($24 \pm 2^\circ\text{C}$) animal housing system with a 12 : 12-h light–dark cycle. Animals were kept on Ova-free diet (Ssniff, Soest, Germany) and had access to sterilized food and tap water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee of Semmelweis University.

Sensitization and airway challenge

Mice were sensitized by intraperitoneal injection of 20 μg Ova emulsified in 2.25 mg aluminium hydroxide (Imject Alum) in a total volume of 100 μL phosphate-buffered saline (PBS) on days 1 and 14 (Fig. 1). Non-sensitized animals received Alum and PBS only. On days 28, 29 and 30, mice were placed in a plastic chamber and challenged with 1% Ova aerosol (in PBS) or PBS only for 20 min by a compressor nebulizer (Laborex-Sanescor, Vienna, Austria). This allergization protocol was used by us previously [25].

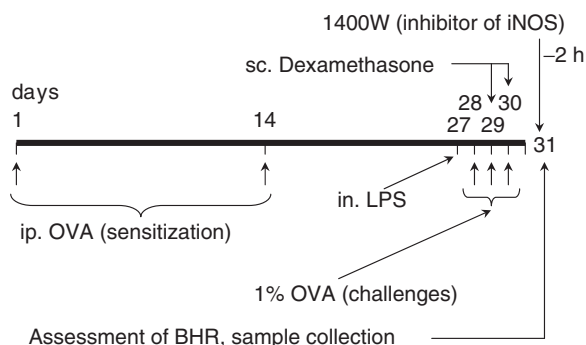


Fig. 1. Protocol of experiments II and III.

Lung resistance (R_L) measurements and sample collections were timed on day 31, 24 h after the last challenge.

Lps priming

Mice were treated with Lps on day 27, 1 day before the first Ova (or PBS) challenge (4 days before measurements of lung resistance, BAL and sacrifice, Fig. 1). After anaesthesia by diethyl ether in a vapour-filled jar, 10 μg Lps (167 $\mu\text{g}/\text{mL}$, $5 \times 10^4 \text{ EU}$ – Limulus lysate assay) from *Escherichia coli* (serotype 055:B5) was instilled intranasally as described by Szarka et al. [26]. Sixty microlitres of Lps solution was placed onto the nares in three 20 μL drops with a repeater pipette. Non-primed animals received 60 μL PBS. The priming dose of Lps was selected to induce temporary bronchial inflammation during the time of Ova challenges [13, 26].

Steroid treatment

Two groups of mice were treated with dexamethasone sodium phosphate (Dex, Oradexon, Oss, the Netherlands) on days 29 and 30. Dex was diluted in normal saline and 5 mg/kg/day was injected subcutaneously in a total volume of 100 μL . This dosage was previously shown to inhibit all features of allergic airway disease in mice [27]. Other groups received 100 μL normal saline as placebo.

Treatment with 1400W, a selective inhibitor of iNOS

N-(3-(Aminomethyl) benzyl) acetamidine (1400W), a highly selective inhibitor of iNOS [24], was administered subcutaneously at 2 mg/kg dose, 2 h before R_L measurements on day 31 in two groups of mice. 1400W was dissolved in PBS and injected in a total volume of 100 μL . The injected amount of 1400W was calculated from the dose used by Garvey et al. [24] in rats and corrected to the metabolic body size of the mice. Other groups received PBS as placebo.

Measurement of R_L

On day 31 (4 days after intranasal Lps or PBS instillation and 24 h after last Ova or PBS challenge), mice were anaesthetized by an intraperitoneal injection of 72 mg/kg pentobarbital sodium (Nembutal, Sanofi, Budapest, Hungary), the left jugular vein was cannulated and tracheostomy was performed. A microsyringe and an infusion pump were connected with the jugular cannule with a Y-shaped connector. Then, the animals were placed into a whole-body plethysmograph and mechanically ventilated at a rate of 150 breaths/min with a tidal volume of 6 mL/kg (Inspira, Harvard Apparatus, Holliston, MA, USA). One port of a four-way connector was attached to the tracheostomy tube and the other two ports were

attached to the inspiratory and expiratory side of the ventilator, respectively. The fourth port was connected with the dry side of the dry/wet differential pressure sensor of the instrument. The wet side of the sensor was attached to an oesophageal pressure cannule completely filled with water and positioned at the level of pleural cavity. In this way, the transpulmonary pressure could be assessed while airflow was calculated from the pressure changes of the plethysmograph by BioSystem XA software (Buxco Electronics, Sharon, CT, USA). R_L was registered continuously during the infusion of MCh in threefold increasing concentrations [28] to assess AHR. The starting dose of MCh was 43 $\mu\text{g}/\text{kg}$, and the consecutive doses were administered in intravenous bolus at 2 min intervals, but only after R_L normalized or showed new baseline (horizontal trace) at $\leq 120\%$ of pre-MCh baseline. This was a non-cumulative administration, and peak responses of R_L during each infused dose of MCh were considered as representative.

Bronchoalveolar lavage

BAL was performed in each mouse via a tracheostomy tube using 600 μL PBS three consecutive times. The recovery of BAL fluid (BALF) was $81.9 \pm 0.7\%$. BALF was centrifuged at 700 g for 10 min at 4 $^{\circ}\text{C}$, and supernatants were aliquoted, immediately frozen and maintained at -80°C until cytokine and nitrite/nitrate measurements. Cell pellets were resuspended in 900 μL PBS, and 300 μL of cell suspension was added to 300 μL trypan blue stain (0.4%) and the total cell count was determined under a light microscope using a Bürker haemocytometer (MOM, Budapest, Hungary). The remaining cell suspension was diluted with PBS and centrifuged in a cyto-chamber (Hettich Zentrifugen, Tuttlingen, Germany). Slides were stained with a Quick Panoptic hematological dye, and differential cell counts were determined under a light microscope by counting at least 300 cells. The cell types were identified according to the standard morphology.

Histology

After BALF collection, the chest wall of the animals was opened, and the lungs were perfused via the right ventricle with 3 mL PBS to remove residual blood. Lungs were inflated with a fixative (4% phosphate-buffered formaldehyde, pH 7.4) and removed from the thoracic cavity. The left lung was immersed in a fixative for 24 h, and then dehydrated, embedded in paraffin block and 5 μm sections were stained with haematoxylin and eosin for light microscopy examination. The density of perivascular and peribronchial eosinophilic infiltration was evaluated by a semi-quantitative score (0–5), as previously described by Morse et al. [29]. Zero was defined as the absence of any visible eosinophil staining; 1 as the presence of a few scattered eosinophils around airways and blood vessels; 2

as the presence of frequent eosinophil infiltration, with nearly all the eosinophils being identified in a single cell layer surrounding the airways and/or the blood vessels; 3 as eosinophil infiltration of a moderate density within a band at least 10 μm wide surrounding at least one-third of the airways and blood vessels; 4 as abundant eosinophil infiltration of most of the airways and blood vessels, but with clear separation of the infiltrates between most of the airways and blood vessels; and 5 as abundant eosinophil infiltration with lack of separation between the airway and perivascular infiltrates in at least one-quarter of the airways. Histological scoring was performed in a blinded fashion by a qualified pathologist (A. N.).

Nitrate and nitrite measurements

Nitrate and nitrite levels of BALF supernatants were assayed by capillary electrophoresis with transient isotachopheresis preconcentration. This new method was validated and described by us recently [30]. The lower limit of detection was 0.2 and 0.3 μM for nitrate and nitrite, respectively. BALF nitrite concentration was considered as a marker of pulmonary NO synthesis, as the primary decomposition product of NO in an aqueous solution containing oxygen is nitrite, and its further oxidation to nitrate requires the presence of oxyhaemoproteins [31]. BALFs were not contaminated by erythrocytes, and the supernatants were rapidly separated from cells. Animals had free access to food containing nitrate and tap water, and the nitrate concentrations were equally high in BALF of all groups.

Cytokine measurements

The levels of IL-4, IL-5, IL-13, IFN- γ , TNF- α and granulocyte-monocyte colony-stimulating factor (GM-CSF) in BALF supernatants were assessed by commercially available ELISA kits. The assays were performed according to the instructions of the manufacturers. The detection limit was 3 pg/mL for IL-4, 5 pg/mL for IL-5, 1.5 pg/mL for IL-13, 8 pg/mL for IFN- γ , 4.5 pg/mL for TNF- α and 3 pg/mL for GM-CSF. Preliminary tests revealed that mucus interfered with ELISAs and low albumin concentration resulted in high aspecific binding. Therefore, supernatants were first filtered through a cotton gauze plug in centrifuge to remove mucus [32] and one part of 10% bovine serum albumin (BSA) was added to nine parts of supernatant to make 1% BSA concentration in samples. Dilution was taken into account at final calculations. Recovery of internal standards indicated no effect of preparation process on cytokine results.

Design of the experiment

The experiment was performed in three separate runs (I–III):
I: Lps-induced inflammation and AHR ($n = 8/\text{group}$).

Groups of mice:

- CLPS: Control, PBS instilled 24 h before measurements and sample collection;
- LPS 24 h: Lps instilled 24 h before measurements and sample collection;
- LPS 96 h: Lps instilled 96 h before measurements and sample collection.

II: Effects of Lps priming on airway inflammation, AHR and steroid responsiveness in allergized mice ($n = 12\text{--}14/\text{group}$).

Groups of mice:

- C: control, non-sensitized, non-primed and PBS challenged;
- LPS: non-sensitized, Lps-primed (96 h before assessment of R_L and sample collection) and PBS challenged;
- OVA: Ova sensitized, non-primed and Ova challenged;
- LPS/OVA: Ova sensitized, Lps primed and Ova challenged;
- OVA+DEX: Ova sensitized, non-primed, Ova challenged and treated with Dex (on the second and third days of Ova challenges);
- LPS/OVA+DEX: Ova sensitized, Lps primed, Ova challenged and treated with Dex (on second and third day of Ova challenges);

All OVA and LPS/OVA mice were injected with normal saline subcutaneously (as placebo) at the time of Dex treatments of OVA+DEX and LPS/OVA+DEX mice. Cellular build-up, cytokine, nitrite and nitrate concentrations of BALF and lung histology were examined in all mice. AHR was assessed in half of the mice in each group (randomly selected, $n = 6\text{--}8$).

III: Short-term effects of 1400W on the AHR. ($n = 6\text{--}8/\text{group}$).

Groups of mice:

- C, LPS, OVA and LPS/OVA groups as described above (II), except that the latter two groups received placebo (PBS) at the time of 1400W treatments;
- OVA+1400W: Ova sensitized, non-primed Ova challenged and treated with 1400W (2 h before assessment of R_L and sample collection);
- LPS/OVA+1400W: Ova sensitized, Lps primed, Ova challenged and treated with 1400W (2 h before assessment of R_L and sample collection).

Cellular build-up of BALF was examined and AHR was assessed in all these mice. There were no significant differences between either BALF cell counts or AHR results of C, LPS, OVA and LPS/OVA groups of the II and the III experiment, respectively. Therefore, these data were drawn together.

Statistical analysis

Parametric results are expressed as mean $\pm$ SEM, and non-parametric data (histological scores) as median and inter-quartile range (in parentheses). One-way analysis of variance (ANOVA) was used for parametric and the Kruskal–Wallis test for non-parametric variables to determine overall difference between groups. Pair-wise comparisons were performed by Neumann–Keuls and Dunn's *post hoc* test as appropriate.

Materials

IL-4, IL-5, IFN- γ , TNF- α and GM-CSF ELISA kits were purchased from Bender MedSystems (Vienna, Austria) and IL-13 from R&D (Minneapolis, MN, USA). The chemicals were used as follows: Ova (chicken egg albumin, Grade V), lipopolysaccharide (*E. coli*, serotype 055:B5), methacholine (acetyl- β -methylcholine), trypan blue dye and BSA (bovine serum albumin) from Sigma-Aldrich (St. Louis, MO, USA); aluminium hydroxide (Imject Alum) from Pierce Biotechnology (Rockford, IL, USA); Dex sodium phosphate; *N*-(3-(Aminomethyl) benzyl) acetamide (1400W) from EMD Biosciences (San Diego, CA, USA); pentobarbital sodium; and Quick Panoptic haematological dye from Cypres (Langdorp, Belgium).

*Results**Airway inflammation*

Intranasal instillation of Lps induced severe, but temporary neutrophil airway inflammation and hyper-responsiveness lasting 1–2 days. Ninety-six hours after Lps (in LPS 96 h group), moderately increased numbers of macrophages and some neutrophils were found in BALF, but at that time AHR had subsided (Fig. 2).

In OVA mice, 1 day after the Ova challenge, a marked increase in BALF eosinophil and macrophage cell counts was observed. In LPS/OVA mice, the presence of macrophages and lymphocytes, but especially eosinophil granulocytes, became more pronounced than in OVA mice. This indicated that Lps instillation 1 day before a 3-day Ova challenge significantly potentiated the eosinophil character of airway inflammation. The semi-quantitative assessment of lung eosinophil infiltration showed that in LPS mice, some eosinophils occurred around airways and blood vessels, but the scores were not significantly different from controls (C: 0.5 (0–1); LPS: 2 (1–2); $P > 0.05$ C vs. LPS). In OVA and LPS/OVA groups, marked eosinophil infiltrations were found (OVA: 3.5 (3–4); LPS/OVA: 4 (3–4); $P < 0.05$ or less vs. both C and LPS).

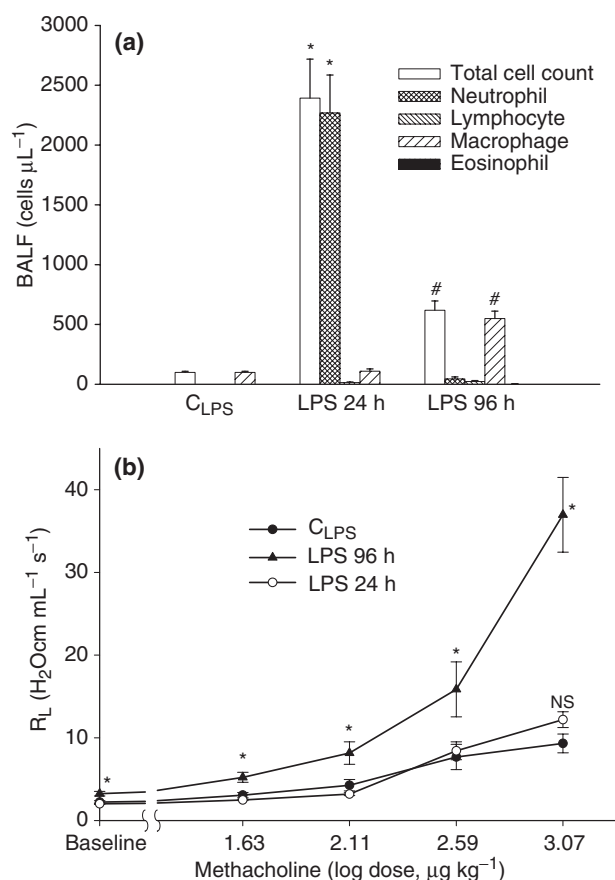


Fig. 2. Experiment I. (a) Total and differential cell counts in bronchoalveolar lavage fluid (BALF) after sham treatment (C_{LPS}), 24 and 96 h after lipopolysaccharide (Lps) treatment (LPS 24 h, LPS 96 h). * P < 0.01 vs. C_{LPS} and LPS 96 h; # P < 0.05 vs. C_{LPS} and LPS 24 h (n = 8/group). (b) Effects of Lps on airway hyper-responsiveness (AHR) (elevation of lung resistance (R_L)) in the same groups. AHR was present at 24 h after Lps treatment (LPS 24 h), but it had normalized after 96 h (LPS 96 h). * P < 0.01 LPS 24 h vs. both C_{LPS} and LPS 96 h (n = 8/group). The baseline R_L values were also increased (P < 0.01) in LPS 24 h (3.25 $\pm$ 0.28) vs both C_{LPS} (2.23 $\pm$ 0.16) and LPS 96 h (2.02 $\pm$ 0.08).

Nitrate and nitrite

The concentrations of nitrite were found to be different among the groups of experiment II. NO₂ was somewhat elevated in LPS, more increased in OVA and the highest in LPS/OVA mice (Fig. 4). Therefore, Lps priming exaggerated NO₂ formation in the lung after Ova challenges. BALF nitrate concentrations were not different among these groups (the overall mean $\pm$ SEM of C, LPS, OVA, LPS/OVA, OVA+DEX and LPS/OVA+DEX was 10.46 $\pm$ 0.3 μM).

Cytokines

As expected, IL-4, IL-13, as well as IL-5 concentrations were markedly increased in OVA mice (Fig. 5). In LPS/OVA counterparts, however, IL-4 and IL-13 were reduced relative to OVA animals, while IL-5 remained unchanged.

BALF concentration of IFN- γ , GM-CSF and TNF- α remained below the detection limit in all groups. Induction of a T-helper type 1-polarized response by Lps could not be demonstrated at this time-point (96 h after Lps instillation).

Airway hyper-responsiveness

As compared with C, there was no increase in airway responsiveness to intravenous infusion of MCh 96 h after Lps treatment in the LPS group (Figs 2b and 6a). AHR in OVA mice was, however, striking, as R_L increased four times more than in C or LPS animals after the two largest doses of MCh. This demonstrated the development of the known AHR of mice undergoing the described protocol of sensitization and challenge. AHR became reduced by Lps priming in Ova-sensitized and challenged mice, as elevation of R_L during infusion of MCh was observed to be midway between OVA and C (or LPS) mice (Fig. 6a).

Effects of dexamethasone

Dex effectively reduced eosinophil inflammation of OVA mice (OVA+DEX, Fig. 3). In LPS/OVA animals, treatment with Dex also led to reduced cellular inflammation, but the absolute number of eosinophils and macrophages remained significantly higher than in OVA+DEX mice.

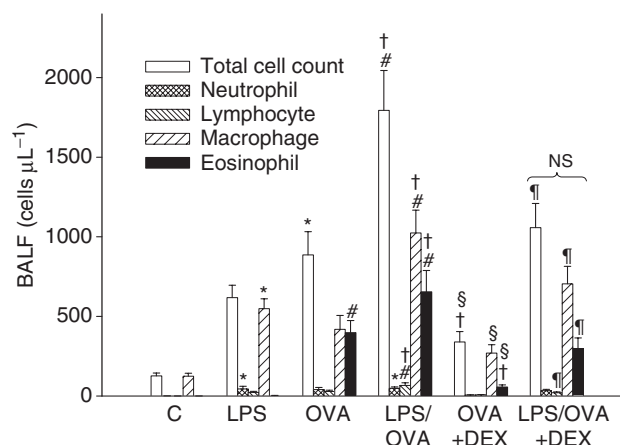


Fig. 3. Experiments II and III. Total and differential cell counts in bronchoalveolar lavage fluid (BALF). Eosinophil inflammation was aggravated by lipopolysaccharide (Lps) priming in ovalbumin (Ova)-sensitized and challenged (LPS/OVA) animals. Treatment with dexamethasone (Dex) (5 mg/kg/day) substantially reduced eosinophil and macrophage counts in OVA mice (OVA+DEX). Dex in LPS/OVA+DEX mice reduced inflammatory cell counts to the level observed in untreated OVA animals. * P < 0.05 vs. C; # P < 0.01 vs. C and LPS; † P < 0.05 vs. OVA; § P < 0.05 vs. LPS/OVA+DEX; ¶ P < 0.05 vs. LPS/OVA; NS: P > 0.05 vs. OVA in all cell populations. (There were no significant differences between BALF cell counts of C, LPS, OVA and LPS/OVA groups of experiments II and III, respectively. Therefore, these data were drawn together; n = 18–20 in C, LPS, OVA and LPS/OVA; n = 12–14 in OVA+DEX and LPS/OVA+DEX).

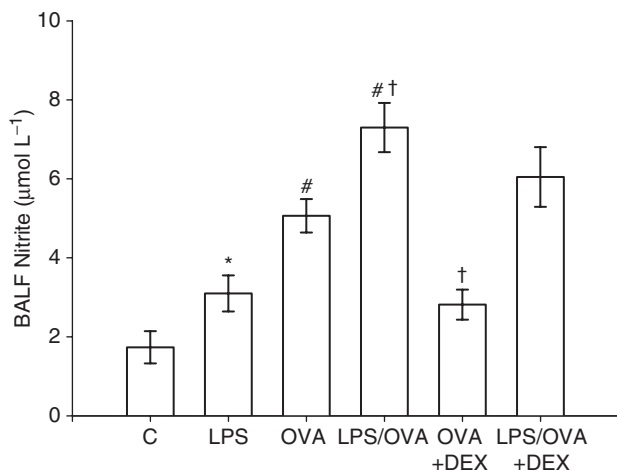


Fig. 4. Experiment II. The nitrite concentration of bronchoalveolar lavage fluid (BALF) was elevated 96 h after intranasal instillation of Lps (LPS). Ovalbumin (Ova)-sensitized and challenged mice (OVA) had greater nitrite levels than C and LPS. Combination of the two treatments (LPS/OVA) generated the most pronounced increase in nitrite concentration. Dexamethasone (Dex) reduced BALF nitrite in Ova-allergized animals (OVA+DEX), but failed to modify it in Lps-primed allergized mice. * $P < 0.01$ vs. C; # $P < 0.01$ vs. LPS; † $P < 0.01$ vs. OVA ($n = 12-14$). Lps, lipopolysaccharide.

Of note, in LPS/OVA+DEX animals, the size of these inflammatory cell populations was as high as in untreated OVA animals. The semi-quantitative eosinophil infiltration score of histological preparations supported the observations made in BALF (OVA+DEX: 1 (1-1); $P < 0.001$ vs. OVA; LPS/OVA+DEX: 2 (2-2); $P < 0.05$ vs. C and LPS/OVA). Similar eosinophil infiltration scores were revealed in LPS/OVA+DEX and OVA animals ($P > 0.05$). The NO_2 concentration in BALF was effectively reduced by Dex in OVA+DEX mice ($P < 0.05$ vs. OVA), while no significant effect was revealed in LPS/OVA+DEX animals ($P > 0.05$ vs. LPS/OVA, Fig. 4). In contrast to cellular inflammation and nitrite levels, the concentration of all three Th2 cytokines became proportionally reduced by Dex in both OVA+DEX and LPS/OVA+DEX mice (Fig. 5). Dex markedly inhibited AHR of mice with pure allergic inflammation (OVA+DEX), while there was only a non-significant trend towards benefit of Dex on AHR of LPS/OVA+DEX (Figs 6b and c).

Effects of 1400W

Figures 6b and c also depict R_L data of OVA+1400W and LPS/OVA+1400W mice. These animals received one dose of 1400W, just 2 h before measurements. This compound was found to be a more effective inhibitor of AHR than Dex in OVA mice (Fig. 6b). In LPS/OVA animals, however, AHR was as refractory to 1400W, as to Dex (Fig. 6c). Cell counts in BALF were not influenced by 1400W in either groups of mice (data not shown).

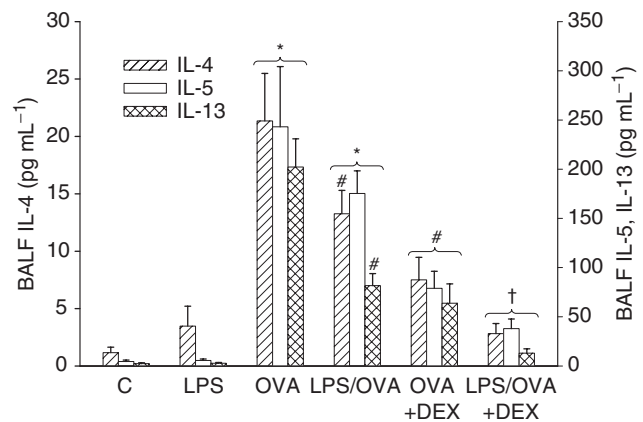


Fig. 5. Experiment II. Concentrations of Th2 cytokines in bronchoalveolar lavage fluid (BALF) supernatants. The amounts of IL-4 and IL-13 were diminished by lipopolysaccharide (Lps) priming in ovalbumin (Ova)-sensitized and challenged (LPS/OVA) animals, compared with OVA mice, while IL-5 remained unchanged. All cytokines were reduced proportionally by dexamethasone (Dex) in both OVA+DEX and LPS/OVA+DEX animals. * $P < 0.05$ vs. C and LPS; # $P < 0.05$ vs. OVA; † $P < 0.05$ vs. LPS/OVA ($n = 12-14$).

Discussion

According to clinical observations made in atopic asthmatics, symptoms may be frequently related to bronchial inflammation induced by mixed aetiologies, e.g. concomitant allergen and Lps challenge of the airways [2, 33-35]. The interaction between Lps and allergic airway inflammation has, however, remained controversial, as Lps, when given around the time of sensitization, blocked the development of experimental asthma [16, 19]. These observations seemed to support the hygiene hypothesis. Among other cytokines, $\text{IFN-}\gamma$ is produced during inflammation induced by Lps [4]. Via antagonism between Th1 and Th2 cytokines, early administration of Lps might suppress the development of allergy [36]. In clinical asthma, the protective role of early exposure to Lps is not so evident, as a recent survey of infants (0-1 years of age) with familial predisposition to asthma or allergy indicated that home endotoxin exposure was an independent risk factor for any or repeated wheeze [35]. Some experimental studies, including the present one, also support synergism between allergen and Lps in the induction of Th2-driven allergic airway disease [4, 5, 37]. Moreover, Lps was reported to enhance the synthesis of allergen-specific IgE [37], and the targeted deletion of Lps-binding protein or toll-like receptor-4, the cell membrane receptor of Lps, prevented the inducibility of experimental asthma in mice [5, 38].

Lps inhalation may trigger symptoms in established asthma as well [2, 7]. In addition, among other causes [39], inhalation of Lps by allergic asthmatic individuals may be responsible for reduced responsiveness to steroids. Michel et al. [11] reported that inhalation of Lps by human

volunteers was followed by a steroid refractory acute phase response of plasma proteins. Trapp et al. [12] investigated how inhalational or systemic (i.v.) steroids prevented corn dust extract-(largely Lps-)induced reduction of forced expiratory volume in 1 s. They have shown that bronchoconstriction was not or only marginally reduced by steroids. Lefort et al. [13] instilled mice with

Lps intranasally. The resultant bronchial inflammation and hyper-responsiveness were completely refractory to Dex. Thus, exposure of airways to Lps concomitant with an allergen challenge may be one cause of reduced treatability by steroids. This has not yet been studied. Our experimental protocol has established a model of eosinophilic bronchial inflammation and AHR induced by Lps plus allergy. In previously published similar studies, Lps administration to allergic rats [16] or mice [15] resulted in airway neutrophil inflammation and blunting of the eosinophil nature of allergic inflammation. As in clinical asthma, airway inflammation is dominated by eosinophil granulocytes, our mouse model may be more appropriate. In fact, LPS/OVA mice had more severe eosinophil inflammation than their OVA counterparts. As BALF concentration of IL-5 was not higher in LPS/OVA than OVA mice, other mediators of tissue eosinophil accumulation, like the LPS-inducible eosinophil chemotactic agent acting through the CCR3 receptor [40], might have been involved. This and several other pro-inflammatory actions of Lps [4, 15] might explain its variable influence on airway allergy. The non-Th2 (or not only Th2) cytokine-induced lung eosinophil inflammation observed in the present experiment was proved to be less responsive to high-dose steroid treatment. LPS priming of allergized mice might have induced additional inflammatory pathways, as not only eosinophil inflammation, but the synthesis of NO, as well as AHR were observed to be less responsive to Dex treatment. Thus, we have succeeded to establish a state of steroid-resistant allergic airway inflammation in mice.

NO is mostly considered to be pro-inflammatory in allergic airways of humans [20, 41] and mice [22, 42, 43], as well as other species [6, 44–46]. The amount of exhaled NO is elevated in human asthmatics [47] and glucocorticosteroids reduce asthma symptoms as well as exhaled NO [20, 48]. These point to iNOS as the major source of increased NO synthesis in asthmatic airways in humans. In some [23, 43], though not all [42, 49], mouse models, iNOS has also been demonstrated to be overexpressed in allergic airways. According to Xiong et al. [43], iNOS-deficient mice could not be allergized to develop eosinophilic airway inflammation and AHR, a finding not

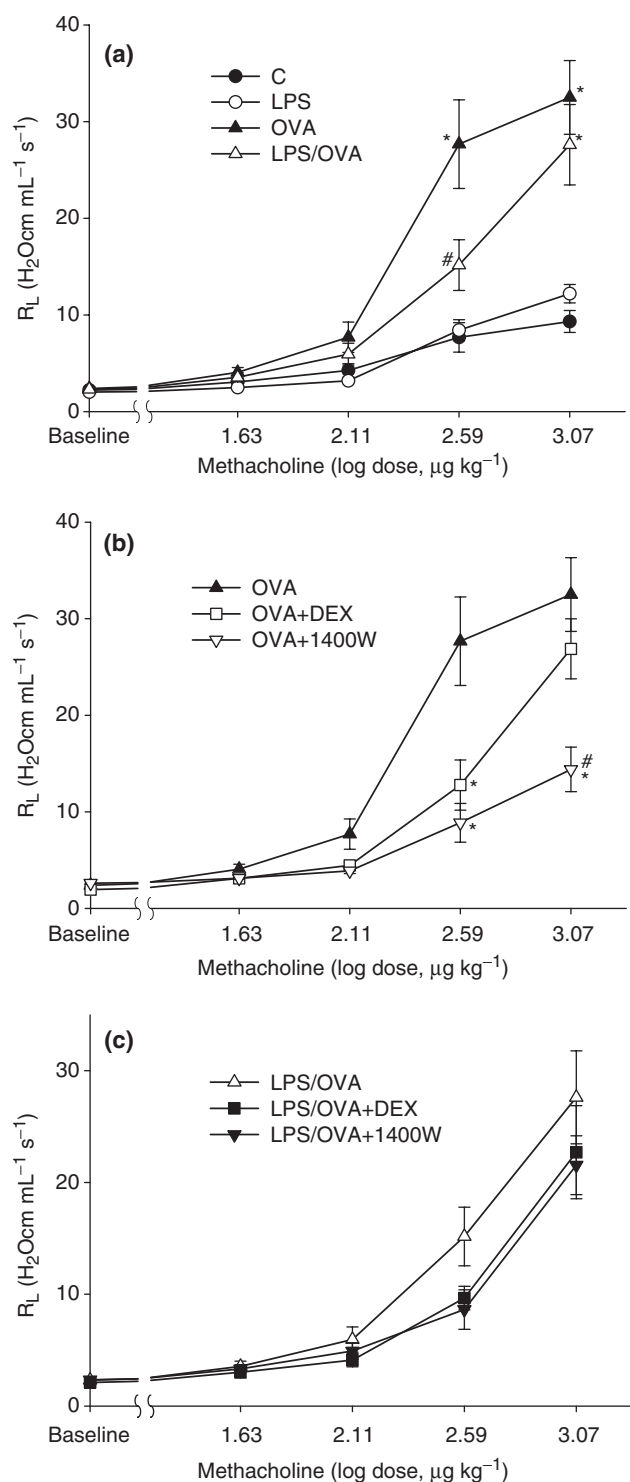


Fig. 6. Experiments II and III. (a) Effects of lipopolysaccharide (Lps) priming on AHR (elevation of lung resistance (R_L)). Lps-primed ovalbumin (Ova)-sensitized and challenged animals (LPS/OVA) were less responsive than OVA mice. * $P < 0.001$ vs. C and LPS; # $P < 0.01$ vs. OVA. (b) Effects of dexamethasone (Dex) and 1400W (inhibitor of iNOS) on airway hyper-responsiveness (AHR) of Ova-sensitized and challenged (OVA) mice. 1400W was more protective against AHR than Dex. * $P < 0.05$ vs. OVA; # $P < 0.05$ vs. OVA+DEX. (c) Effects of Dex and 1400W on AHR of Lps-primed Ova sensitized and challenged (LPS/OVA) mice. AHR was not reduced significantly by either Dex or 1400W ($n = 14-16$ in C, LPS, OVA and LPS/OVA; $n = 6-8$ in OVA+DEX, LPS/OVA+DEX, OVA+1400W and LPS/OVA+1400W).

confirmed by De Sanctis et al. [42]. On the other hand, 1400W, a highly selective inhibitor of iNOS, was also reported to blunt AHR in allergized mice [50]. As glucocorticoids are powerful and selective suppressors of iNOS, and the production of NO was found to be markedly reduced in OVA+DEX mice, our results support the role of iNOS in AHR of pure allergic origin.

We report here, for the first time, that 1400W, a selective inhibitor of iNOS, when given to mice with purely allergic airway inflammation (OVA mice) only once, after the last challenge and just 2 h before lung resistance measurements, reversed AHR even more powerfully than a 2-day long Dex treatment. This confirms earlier observations of Iijima et al. [51] and Koarai et al. [23], who have already reported that longer-term, repeated administration of 1400W exerted concomitant anti-inflammatory and AHR-attenuating effects in mice. Our finding that 1400W normalized AHR within 2 h indicated that to some extent this abnormality might be maintained by short half-life substances formed continuously in diseased airways. For example, peroxynitrite, formed from NO and O_2^- , may be one such molecule [6] and thereby the immediate action of 1400W could be explained by an immediate decrease of peroxynitrite formation within the bronchial wall.

On the other hand, if NO of iNOS origin was the crucial determinant of AHR in LPS/OVA mice, these animals should have had an even greater R_L response to MCh than OVA. The opposite was observed: higher nitrite levels (NO synthesis) were combined with less severe AHR. We have not further investigated the mechanisms of remaining hyper-responsiveness in LPS/OVA animals, but in this particular model another phase of the NO system might have become predominant. Que et al. [52] have recently reported that S-nitrosothiols (SNO), formed by NO from cysteine sulphurs, provided protection against AHR in a similar mouse model. Forthcoming experiments will clarify whether a well-timed Lps exposure of allergic airways can augment the formation of bronchodilator SNOs disproportionately compared with other NO-related, but bronchoconstrictor compounds like peroxynitrite [6].

In conclusion, Lps priming timed before Ova challenges in Ova-sensitized mice induced experimental airway allergy that was characterized by exaggerated eosinophil inflammation and NO production. Airway responsiveness to MCh and synthesis of Th2 cytokines were moderated, but still remained significantly increased. Inflammation and AHR were refractory to treatment by Dex and 1400W. These new observations shed some light on one possible cause (concordant inhalation of Lps) of resistance to anti-inflammatory agents in allergic airway inflammation.

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