

Airborne endotoxin exposure and the development of airway antigen-specific allergic responses

G.-H. WAN,*† C.-S. LI,* and R.-H. LIN†

*Graduate Institute of Environmental Health, College of Public Health and †Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei, Taiwan

Summary

Background and objective Repeated exposure of aerosolized antigen via respiratory tract can induce immunoglobulin (Ig) E isotype-specific tolerance to this antigen. However, the atopic individuals often produce a higher titre of IgE in response to airborne environmental allergens. The mechanisms of this differential regulation of airway allergen-specific immune responses are not fully understood. This study investigated the role of airborne endotoxin on the initiation of antigen-specific airway allergic responses.

Methods ELISA methods for detection of isotypes of antigen-specific antibodies and competitive reverse transcription polymerase chain reaction for detection mRNA of cytokines were used. In addition, Liu stain method was used to analyse the amounts of eosinophils in bronchoalveolar lavage fluid.

Results Mice pre-exposed with airborne endotoxin mounted significantly higher amounts of OVA-specific IgE antibody responses to inhaled OVA than those OVA-only sensitized mice. Inhaled endotoxin could downregulate repeated airway antigen exposure-induced IgE isotype-specific tolerance and increase antigen-induced lung eosinophils infiltration.

Conclusions These data show that airborne endotoxin exposure could potentiate allergen-specific airway inflammation. The results should have potential implications for understanding the development of allergen-induced airway allergic responses.

Keywords: airway inflammation, animal model, bioaerosol, endotoxin

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Introduction

Atopic persons often mount type 2 T helper cell (TH2) and immunoglobulin (Ig) E antibody responses to environmental allergens, in contrast with nonatopic individuals, whose allergen-specific immune reactions are mainly mediated by TH1 and responding antibodies belong to an IgG isotype [1,2]. TH2-mediated immune responses play a very important role in the mediation of allergic responses. The regional cytokine environment at the time of naive T-cell activation is known to be of critical importance in determining the functional differentiation of T cells [3–7]. Until now, the mechanisms of a high TH2 production in response to

environmental allergens in atopic subjects remain to be investigated.

According to an epidemiological study of asthma in schoolchildren in Taipei, the prevalence of asthma increased from 1.3% in 1974 to 10.8% in 1994 (unpublished data). The predominant perennial allergen in Taiwan is house dust mite (HDM) [8,9]. However, the levels of mite allergen in this subtropical area are always found to be higher than the threshold for sensitization of HDM allergic responses [8,9]. Thus, the amounts of mite allergen exposure may not be the determining factor for allergy sensitization [8–11]. Furthermore, repeated inhalation of protein antigen can lead to the suppression of allergen-specific IgE responses in humans as well as mice [12–15]. Therefore, the reason why inhaled allergen can initiate persistent IgE responses in allergic individuals remains to be investigated. It is reasonable to assume that other environmental contaminants may play an

Correspondence: R.-H. Lin, Dr rer. nat., Graduate Institute of Immunology, College of Medicine, National Taiwan University, No. 1, Jen Ai Road, 1st Sect. Taipei, Taiwan (e-mail: rhlin@ha.mc.ntu.edu.tw).

important role in turning on airway antigen-specific IgE-mediated allergic responses.

From many previous investigations [16–18], it was indicated that aerosolized endotoxin can not only cause an inflammatory response in the airway but also play a certain role in chronic asthmatic diseases [18]. By using a murine model, we directly investigated the effects of endotoxin on the generation of antigen-specific airway allergic responses. It demonstrated that inhaled endotoxin can potentiate airway antigen-specific allergic immune responses. These results should have potential implications for understanding the development of allergen-induced airway allergic responses.

Materials and methods

Animals, endotoxin and antigen

Female BALB/cJ mice, aged between 6 and 8 weeks, were obtained from the animal-breeding centre of College of Medicine, National Taiwan University. The animal experiment confirmed to NIH guidelines (Guide for the Care and Use of Laboratory Animals, NIH Publication no. 86–23, Revised 1985, US Government Printing Office, Washington DC 20402–9325). Lipopolysaccharide (*Escherichia coli* 026 B6, Difco Lab, Detroit, MI, USA) was used as endotoxin in the study. The endotoxin was suspended in pyrogen-free water (40 ng/mL). Antigen was 5x recrystallized chicken ovalbumin (Sigma, St Louis, MO, USA) dissolved in PBS (10 mg/mL).

Endotoxin exposure

The aerosols of the endotoxin solution were generated by a DeVilbiss 464 nebulizer (DeVilbiss, Somerset, PA, USA) driven by compressed air at 8 L/min with 0.3 mL/min output at a continuous pressure of 12 psi with particles in the 0.5–5 µm size range and transmitted into a 25-cm diameter exposure chamber 10 cm in height. The exposure endotoxin concentration was 40 ng/mL. The mice were exposed with endotoxin for 40 min.

Preparation of lung wall cells for detection of cytokine mRNA expression by competitive reverse transcription polymerase chain reaction (RT-PCR)

The lungs were cut in thick slices and suspended in plastic Petri dish containing 3 mL of complete culture medium (RPMI 1640) supplemented with penicillin (100 Units/mL), streptomycin (100 µg/mL), L-glutamine (2 mmol/L), 2-mercaptoethanol (5×10^{-5} mol/L), and 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, USA) with 0.2 mg/mL collagenase (Sigma) and 30 µg/mL DNase (Sigma).

Following an incubation of 60 min in a 37-g/shaking water bath, red blood cells were lysed with RBC lysis buffer (17 mmol/L TRIS-HCl pH 7.2 containing 0.144 mol/L ammonium chloride). The cells were collected by centrifugation and referred to as lung wall cells. The lung wall cells were harvested for cytokine mRNA determination by competitive RT-PCR. The competitive RT-PCR technique was modified from our previous study [19]. PCR was performed in an automatic DNA thermal cycler (Perkin Elmer, Norwalk, CT, USA) as follows: denaturation 94 °C, 40 s, annealing 55 °C, 1 min, extension 72 °C and 1 min.

Immunizations

For airway immunization, the mice were first exposed to 40 ng/mL of endotoxin for 40 min and sensitized subsequently (18 h later) with a daily 20-min 10 mg/mL of OVA exposure for two 5-day periods with a 40-min endotoxin exposure in between. Fourteen days later, the mice were re-exposed to 10 mg/mL of OVA for 5-day periods. The sera were obtained 7 days later and stored at –20 °C until analysis. Age-matched control mice were exposed to OVA alone with the same protocol. Two weeks after the last aerosol exposure the animals were challenged by intraperitoneal (i.p.) injection with 10 µg of OVA dissolved in 10% aluminium potassium sulphate and the sera were analysed 7 days later.

Determination of OVA-specific IgG2a and IgE Levels by ELISA

The methods for detection of antigen-specific IgE and IgG2a have previously been described [19]. In brief, for determining IgE levels, 96-well vinyl microtitre plates (Costar, Cambridge, MA, USA) were coated with 50 µg/mL of OVA diluted in NaHCO₃ buffer with pH 8.2. After overnight incubation at 4 °C, plates were washed two times with 0.1 mol/L TRIS–Tween20 (0.05%, v/v) and blocked with TRIS–BSA (1%, w/v) buffer, pH 9.6, for 2 h at 37 °C. Serum samples were diluted in 0.1 mol/L TRIS–BSA–1% Tween20 buffer with a 1:20 dilution, added in the plates and incubated overnight at 4 °C. Plates were then washed, and alkaline-phosphatase-conjugated monoclonal rat anti-mouse IgE (PharMingen, San Diego, CA, USA), diluted in TRIS–BSA–1% Tween, buffer was added for an additional 2 h. The reaction was developed with amplification system (Life Technologies, Gaithersburg, MD, USA). Plates were read using a microplate reader (Metertech, Taipei, Taiwan) at 490 nm. For IgG2a detection, the plates were freshly coated with OVA at a concentration of 10 µg/mL in NaHCO₃ buffer, pH 8.2. The plates were developed with alkaline-phosphatase-conjugated monoclonal rat anti-mouse IgG2a system (PharMingen). The plates were read in microplate reader

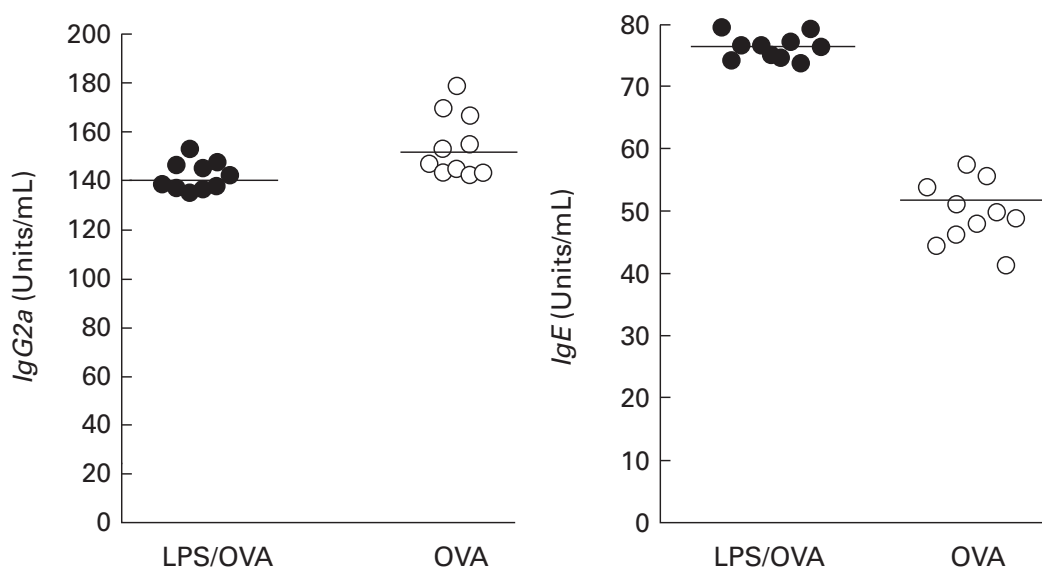


Fig. 1. The effect of endotoxin in humoral immune responses to airway exposed antigen. Female BALB/cJ mice ($n = 10$) were first exposed with 40 ng/mL of endotoxin for 40 min, after 18 h, the sensitization was performed with a daily 20-min 10 mg/mL OVA exposure for two 5-day periods with a 40-min endotoxin exposure in between, during the first airway sensitization. After 14 days, the mice were re-exposed to 10 mg/mL of OVA for 5-day periods. The sera were obtained 7 days later and stored at -20°C analysis. Age-matched control mice ($n = 10$) were exposed to OVA alone with the same protocol. Each symbol represented the data derived from individual serum. ELISA experiments were assayed in duplicate. The median value of each group is presented as the symbol ‘-’.

(Metertech) at 405 nm. Results are expressed as ELISA units relative to an internal isotype standard run in each assay. One ELISA unit is equivalent to the response of 1 ng/mL of purified isotype standard antibody.

Evaluation of eosinophils in bronchoalveolar lavage (BAL) fluid

Two weeks after the last OVA antigen exposure, the mice were challenged with aerosolized OVA (10 mg/mL). After 24 h, the complete culture medium was slowly injected into the bronchus. The BAL fluid was collected in centrifuge tubes and placed on ice. The lavage cells were centrifuged and resuspended in complete culture medium. A small sample of the cell suspension was centrifuged (Cytospin; Shandon Southern Instruments, Pittsburgh, PA, USA) and approximately 30 g cells were spun at 500 r.p.m. for 8 min onto a glass slide. The cells were air-dried and stained with Liu (Delta, Taipei, Taiwan) for differential count of 300 cells per slide by light microscopy.

Statistical analysis

The results were analysed for their statistical significance by Wilcoxon Rank-Sum test for Figs 1 and 4, and Kruskal-Wallis test for Figs 2 and 3, and $P < 0.05$ was taken as the level of significance.

Results

Airborne endotoxin enhances antigen-specific IgE responses

To study whether airborne endotoxin has an enhancing effect on the antigen-specific IgE response, the mice were inhaled with endotoxin and OVA antigen. As shown in Fig. 1, mice pre-exposed with airborne endotoxin mounted significantly ($P < 0.01$) higher amounts of OVA-specific IgE antibody responses to inhaled OVA than those OVA only sensitized mice. On the contrary, the OVA-specific IgG2a antibody response was significantly reduced in the endotoxin/OVA-sensitized group compared with those of the OVA alone sensitized group ($P < 0.01$).

Endotoxin can inhibit airway antigen-induced IgE tolerance

We further evaluated that the effect of airborne endotoxin on the generation of inhaled antigen-induced IgE-isotype-specific tolerance. As shown in Fig. 2, repeated airborne OVA exposure downregulated OVA-specific IgE responses to i.p. immunized OVA antigen ($P < 0.01$). On the contrary, the mice receiving airborne endotoxin exposure before repeated OVA inhalation mounted higher amounts of IgE and lower IgG2a responses to i.p.-immunized OVA than those of no endotoxin treated mice ($P < 0.01$). These results indicated that inhaled endotoxin could suppress airway OVA-induced IgE isotype-specific tolerance.

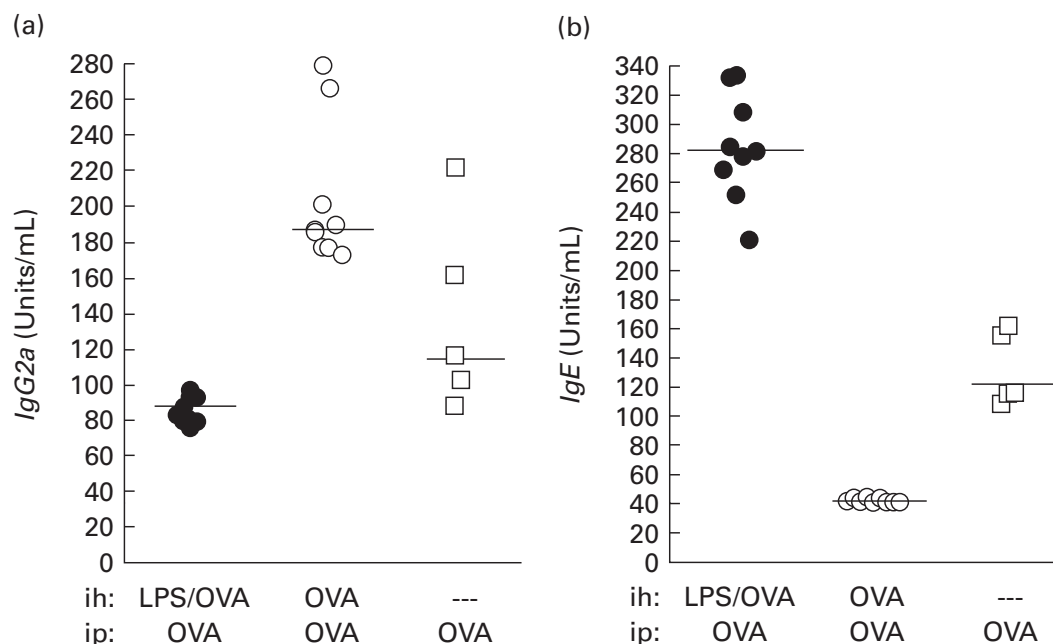


Fig. 2. Endotoxin can inhibit airway antigen-induced IgE isotype-specific tolerance. The animals ($n=9$) were exposed to endotoxin and OVA antigen with the same protocol as described in Fig. 1. Two weeks after the last OVA antigen exposure, the animal were challenged by i.p. injection with $10\ \mu\text{g}$ of OVA dissolved in 10% aluminium potassium sulphate and 7 days later the sera were analysed. Age-matched control mice ($n=5$) were only injected by i.p. with OVA. OVA-specific (a) IgG2a and (b) IgE antibodies were analysed by ELISA. Each symbol represented the data derived from individual serum. Data are representative of those from the two experiments. The median value of each group is presented as the symbol ‘-’

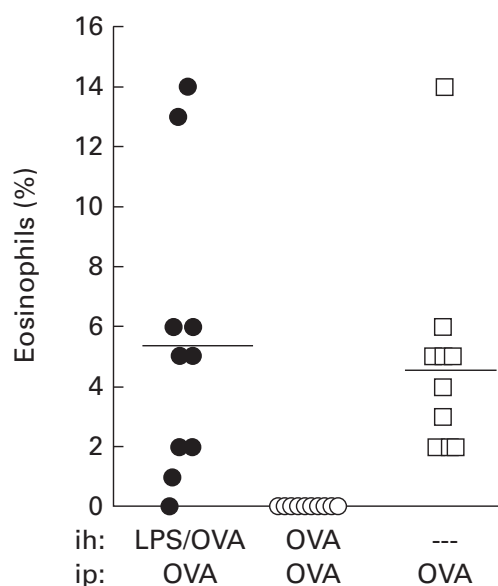


Fig. 3. Endotoxin increased the antigen-induced eosinophil infiltration in antigen-repeated exposed mice. The animals ($n=10$) were treated with the same protocol as described in Fig. 2. Two weeks after the last OVA antigen exposure, the mice were challenged with aerosolized OVA ($10\ \text{mg/mL}$) and 24 h later the BAL fluids were collected. Eosinophils were characterized using Liu's stain. Each symbol represented the data derived from individual BAL fluid. The median value of each group is presented as the symbol ‘-’

Airborne endotoxin increases the antigen-induced lung eosinophils infiltration in antigen repeated exposed mice

To evaluate the influence of airborne endotoxin on the airway allergic responses induced by inhaled antigen, bronchoalveolar lavage cells were stained and counted. After OVA antigen rechallenge, a significant elevation in the number of eosinophils was observed in endotoxin/OVA inhaled mice as compared with those of the OVA-inhaled mice ($P<0.01$) (as shown in Fig. 3). There was no significant difference in the levels of eosinophils between the endotoxin/OVA inhaled and OVA i.p.-only immunized mice.

Endotoxin modulates local expression of IL-10 and IL-12 mRNA by lung wall cells

To investigate the possible role of airborne endotoxin on the regulation of airway antigen-specific immune responses, the TH1/TH2 deviation-related pro-inflammatory cytokines, such as IL-10 and IL-12, were analysed (Fig. 4). Airborne endotoxin exposure induced significantly higher ($P<0.05$) amounts of IL-10 mRNA than those of PBS inhaled control mice. In contrast, airborne endotoxin exposure did not change the synthesis of IL-12 mRNA ($P>0.05$).

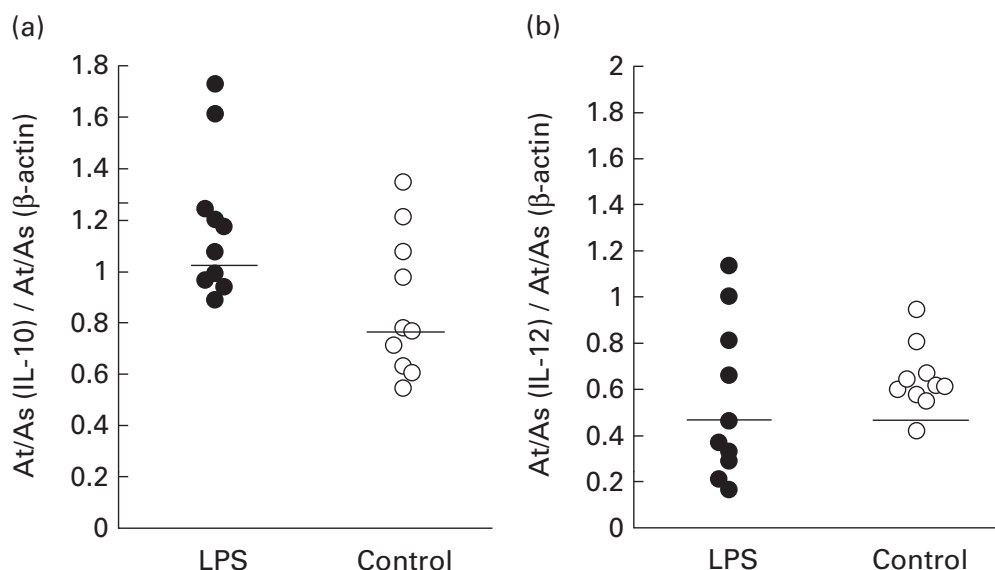


Fig. 4. Airborne endotoxin modulated local expression of (a) IL-10 and (b) IL-12 mRNA by lung wall cells. The mice ($n = 10$) were exposed to 40 ng/mL of endotoxin for 40 min. After 18 h, the lung wall cells were harvested for cytokine mRNA determination. The cells of age-matched control mice were prepared with the same protocol. Following competitive PCR, the amounts of PCR products were semiquantified by densitometer. The ratio of amplified target (experimental cDNA) (At) to amplified standard (PCR MIMICS) (As) PCR products was determined for each sample. The amount of cytokine PCR products was presented in dot plot form after being corrected by the β -actin PCR products. The median value of each group is presented as the symbol '-'

Discussion

We have demonstrated that cytokines induced by inhaled microbial product may play an important role in determining airway antigen-specific T-cell function [19]. The current studies further indicate that aerosolized endotoxin can not only suppress IgE isotype-specific tolerance to repeated exposed airborne antigen, but also promote airway allergen-specific allergic responses in mice. Mice inhaled with endotoxin which subsequently received repeated exposure of aerosolized OVA antigen could mount persistent antigen-specific IgE antibody responses and lung eosinophil infiltration.

CD4⁺ helper T cells are heterogeneous with regard to the production of cytokines and to their functions. TH1 cells secrete IL-2, interferon (IFN) γ , and lymphotoxin [3,20,21], as well as help the induction of IgG2a antibody response in mice [22,23]. In contrast, TH2 cells produce mainly IL-4, IL-5 and IL-10 [3,20,21], and induce IgE response [22–24]. IL-12 and IFN γ have been indicated to be able to promote the development of TH1 cells [4,5,7,25], while the presence of IL-4 and IL-10 greatly favour the development of TH2 cells [6,7,26].

Microbial components, such as endotoxin, are known to be able to activate monocytes/macrophages, which subsequently secrete IL-12 [25,27], IL-6, and IL-10 [28,29]. IL-10 has the ability to inhibit macrophage-derived IL-12 production, thus inhibiting the development of TH1 cells. The

effects of IL-12 on TH1 development are believed to be mediated by subsequently induced IFN γ production by NK cells [25,27]. Thus, the balance between the secretion of IL-10 and IL-12 by the monocytes/macrophages will be crucial in directing the development of TH2 and TH1 cells. In our current study, we demonstrated that inhaled endotoxin can enhance TH2-mediated allergic responses *in vivo*. These results are supported by the finding that LPS enhances IL-10 but not IL-12 production by lung wall cells (Fig. 4).

Currently, the organic dusts and gaseous pollutants are regarded as potential risk factors for allergic diseases [18,19,30,31]. It was suggested that the airway function can be altered by microbial products reaching the lungs via inhalation [32]. Repeated stimulation of mice with aerosolized antigen markedly reduces the antigen-specific IgE responses [13,14]. Our results indicate that the pro-inflammation induced by endotoxin can somehow break this type of tolerance and lead to the sustained production of antigen-specific IgE, and the infiltration of eosinophils. $\gamma\delta$ T cells were shown to play a major role in mediating this type of inhalation-induced IgE isotype-specific tolerance [15]. Whether the effect of airborne endotoxin in the down-regulation of regulatory $\gamma\delta$ T cells is mediated by LPS-induced pro-inflammatory cytokines or cell membrane molecules remains to be investigated.

It has been suggested that virus infection is capable of overriding inhalation-induced IgE isotype tolerance and

promoting airway allergic responses [30]. Endotoxins from the cell wall of Gram-negative bacteria have been also implicated as etiological agents in the induction of acute and chronic respiratory symptoms, including bronchoconstriction and bronchial hyperreactivity [33,34]. In the current study, we demonstrated that airborne endotoxins, which exist frequently in the subtropical area, can also break inhalation-induced IgE isotype tolerance and promote airway-allergic responses. Further understanding of the mechanisms of the endotoxin-induced inflammation in the differential regulation of airway allergen-specific immune responses should provide important insights into the development of human airway allergic diseases.

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