

RESEARCH ARTICLE

Irritant and adjuvant effects of gaseous formaldehyde on the ovalbumin-induced hyperresponsiveness and inflammation in a rat model

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Abstract

Background: Formaldehyde (FA) is a common indoor air pollutant that can cause asthma in people experiencing long-term exposure. While FA and other man-made chemicals contribute to the stimulation of asthma in the general population, the underlying molecular pathogenesis of this relationship is not yet well understood.

Objective: To explore FA as an irritant for the onset of asthma and as an adjuvant for the induction of allergy.

Methods: In the present study, 40 Wistar rats in five experimental groups were exposed to: (i) saline; (ii) ovalbumin (OVA); (iii) OVA + FA at 417 ppb; (iv) OVA + FA at 2500 ppb; and (v) FA at 2500 ppb. Current and prior occupational exposure limits in China were established at 417 ppb and 2500 ppb, respectively. Gaseous FA was administered to the animals for 6 h/day before and during OVA immunization or saline treatment. Measured outcomes included in situ lung function analysis, cytokine measurement, and histological changes in the rat lungs.

Results: The airway reactivity, lung histological changes, pulmonary interleukin-4 secretion, and eosinophil infiltration in the OVA and FA exposed rats were significantly higher after gaseous FA exposures of 417 and 2500 ppb. While FA exposure alone did not induce significant structural changes to the airway, and the rate of inflammatory cell infiltration was the same as for the control group, pulmonary levels of interferon- γ were significantly elevated in the exposed rats.

Conclusions: FA may be an irritant as well as serve as an adjuvant for the onset of asthma or asthma-like symptoms.

Keywords: Asthma; Airway hyperreactivity; Cytokine; Formaldehyde; Rat model

Introduction

Asthma is a chronic inflammatory disorder of the pulmonary airways, characterized by airway hyperresponsiveness (AHR), airway remodeling, and airway inflammation (Marsha et al., 1999). In the last four decades, the incidence of asthma has markedly increased throughout the world, especially in developed countries (Platts-Mills et al., 1997; Bickler et al., 2008). A number of potential causes for this increase include (a) elevated humidity leading to increases in the presence of mites and molds; (b) increased routine immunizations in early life leading to an allergy phenotype; and (c) improvements in the diagnosis and reporting of asthma. Moreover, man-made chemicals and indoor air

pollutants extensively produced in modern society have also been suggested as contributors to the increased incidence of asthma (Becher et al., 1996; Delfino et al., 2002; Ebtekar, 2006; Franchi et al., 2006; Handzel, 2000; Jones, 1998; Piipari et al., 2005). Recently, a series of studies was conducted that aimed to distinguish the effects of these potential environmental influences on the human respiratory system (Andersson et al., 2006; Larsen et al., 2002; Mapp et al., 2005; Nielsen et al., 2007). However, it is difficult to arrive at a convincing conclusion due to continuously changing environmental factors (Gautrin et al., 2003). Furthermore, current limits in both the in vivo and in vitro

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evaluation systems for chemical agents hinder the effectiveness of mechanistic studies (Karol et al., 1994).

In this study, formaldehyde (FA) was adopted as a model exposure for the onset of asthma due to its widespread use in the general environment. FA is also a low-weight chemical linked with the incidence of asthma (Sastre et al., 2003). FA can be released from combustion (e.g., from tobacco smoke and cooking) or from FA-emitting products (such as plywood and furniture) in areas of low ventilation (Mi et al., 2006). Studies have revealed that up to 8233 ppb has been found in rooms where new plywood and/or furniture has been installed (Fang et al., 2005).

FA-induced asthma or asthma-like symptoms have been reported in numerous epidemiologic studies (Burge et al., 1985; Hendrick et al., 1977; Piipari et al., 2005; Rumchev et al., 2002; Smedje et al., 2001; Wieslander et al., 1997). However, the specific mechanism by which FA induces asthma remains elusive (World Health Organization, 2000, 2005). Some studies have indicated immunoglobulin (Ig) E as an important factor for FA-induced asthma. However, FA-specific IgE is rarely detected (Arts et al., 1997; Hilton et al., 1996; Kim et al., 2001; Kim et al., 1999; Krakowiak et al., 1998). This has led to the suggestion that FA may be involved in asthma via a non-IgE-mediated mechanism. As a result, two possible pathways for FA-induced asthma have been proposed: (1) FA alone causes bronchial inflammation or (2) FA acts as an adjuvant for sensitization to common allergens (Mendell, 2007). Exposure to FA can induce transient AHR (Akbar-khanzadeh et al., 1994) or elevate neurogenic inflammation mediators such as substance P (SP) (Liu et al., 2003) and nitrogen oxide (NO) (Franklin et al., 2000) in exposed persons. Likewise, FA has been found to play an adjuvant role to common allergens in animal experiments (Riedel et al., 1996; Tarkowski et al., 1995) and in one epidemiological study (Garrett et al., 1999). Furthermore, FA has been shown to exacerbate allergen-induced asthma symptoms in controlled human exposure experiments, including significant increases in bronchial response and a late-phase reaction to mite allergen (Casset et al., 2006a, 2006b). Nevertheless, in most cases, these two potentially different effects of FA are difficult to distinguish, hence, further studies are necessary.

In this study, an electronically controlled dynamic FA exposure strategy was employed to study the effects of FA exposure in the pathogenesis of asthma.

Materials and methods

Animals

Wistar rats (male, 5–6 wk old and 70–80 g) were purchased from the Hubei experimental animal center (Wuhan, China) and maintained in a pathogen-free room with temperatures 20–25°C and humidity of 50–70%. The experiment was approved by the Office of Scientific Research Management of Huazhong Normal University, with the Certification on Application for the Use of Animals dated December 22, 2006.

Sensitization and challenge protocol

The rats were randomly divided into five groups of eight animals. Figure 1 shows the general allergen exposure protocol for our experimental animal groups, which is similar to what was used in studies by Tarkowski et al. (1995). The rats were administered OVA by intraperitoneal injection on days 10 and 18. This was then followed by an aerosol challenge in 1% OVA (30 min) from days 22 to 28 using an ultrasonic nebulizer (Yuyue 402AI type, China) (group B). The same procedure was carried out for the control group (group A) with saline substituted for OVA. To examine the effect of FA exposure on rat lung function, the same treatment conditions were applied to group B, except the rats were also treated with FA (6 hr/day) from day 0 through 21 at doses of 417 ppb or 2500 ppb. All rats were sacrificed on day 29, at 24 h after the final aerosol challenge.

Preparation of gaseous formaldehyde

FA was prepared from 10% formalin (Sigma, purity >99.99%, USA) and the solution was administered to the rats through a small-scale environmental chamber (WH-2 type, China) capable of emitting gaseous FA at preset concentrations of either 417 ppb or 2500 ppb. Environmental parameters were monitored and controlled automatically by the machine. Air temperature, relative humidity, and airflow rate were maintained at $23 \pm 0.5^\circ\text{C}$, $45 \pm 5.0\%$ and 1.0 L/min, respectively. Experimental animals were placed in a glass chamber connected to the gaseous FA generator. During exposure, concentrations of FA were monitored twice a day using a gaseous FA analyzer (Interscan, 4160-2 type, USA) (Figure 2). According to the occupational threshold limit value in China (Administration of Technology Supervision of People's Republic of China, 2002a, 2002b), high and low exposure levels of FA were adopted at 2500 ppb and 417 ppb, respectively.

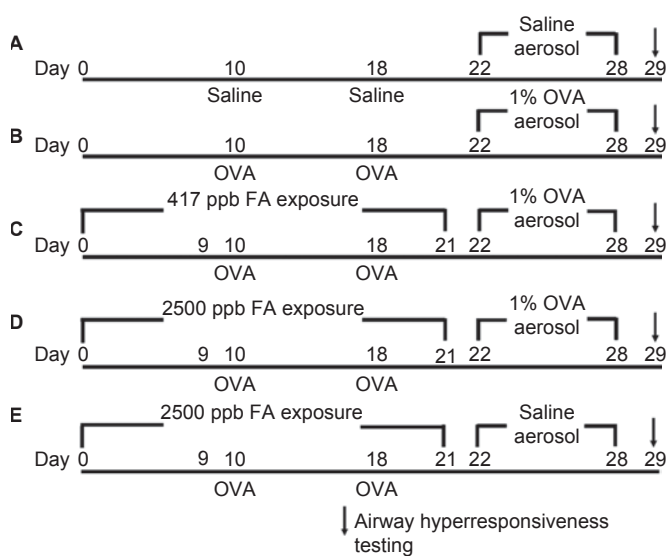


Figure 1. The allergen exposure protocol. (Group A: saline control; Group B: OVA-sensitized group; Group C: 417 ppb FA-OVA group; Group D: 2500 ppb-OVA group; Group E: 2500 ppb FA alone group).

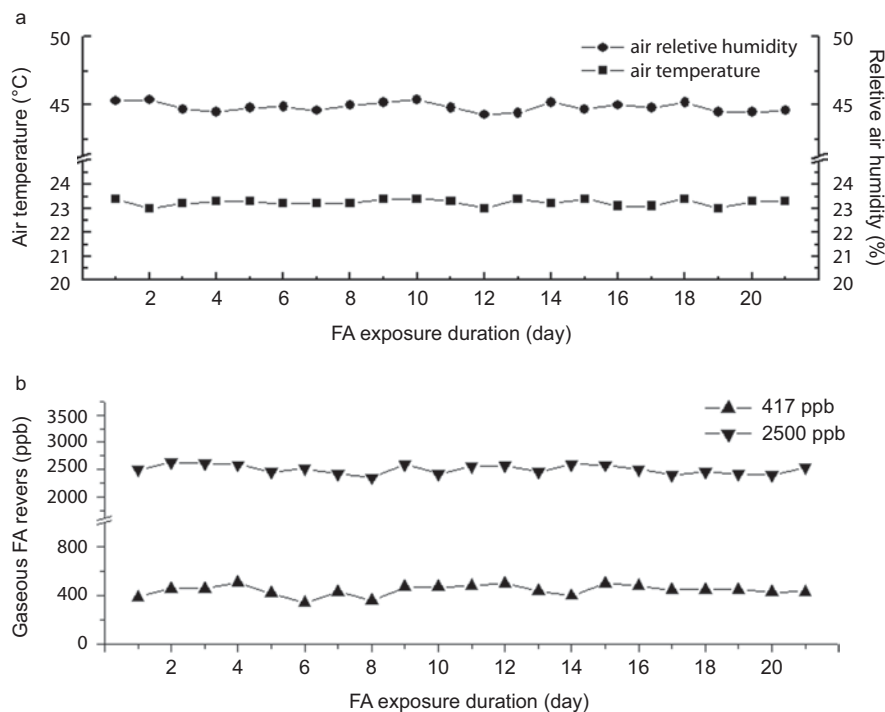


Figure 2. Monitoring of environmental parameters and gaseous FA levels on each day during FA exposure period. A: The air temperature in the exposure chamber was kept at $23 \pm 0.5^\circ\text{C}$ ■; the relative humidity in the exposure chamber was $45 \pm 5.0\%$ ●; B: The concentrations of gaseous FA generated from the small environmental chamber was respectively 417 ± 91.74 ppb ▲ and 2500 ± 150 ppb ▼.

Airway hyperresponsiveness measurements

For each group, airway responsiveness was assessed 24 h after the final aerosol exposure using the AniRes2005 lung function system (Bestlab, AniRes2005, version 2.0, China) according to the manufacturer's instructions (Li et al., 2006). Animals were anesthetized intraperitoneally with pentobarbital sodium (Urchen) (95 mg/kg), and then respiration via a tracheal cannula connected to a computer-controlled small-animal ventilator. The respiratory rate was preset at 75/min and the time ratio of expiration/inspiration at 1.5:1. An injector needle ($\Phi = 0.45$ mm) was then inserted into the jugular vein. Methacholine (*O*-acetyl- β -methylcholine chloride, MCH, Sigma, USA) was administered through a saline-filled catheter with increased dosages of 0.025, 0.05, 0.1, and 0.2 mg/kg at 5-min intervals, and rat airway responsiveness was assessed by indexes of respiratory resistance R-area (the area between the peak value and baseline) (Rrs; including Re and Ri), and the peak value of Cldyn, according to the review by Drazen et al. (1999).

Bronchoalveolar lavage fluid (BALF)

Rats were lavaged with 3 aliquots of 3 ml saline. Cell samples were concentrated by centrifugation (1000 rpm for 10 min at 4°C) and resuspended in 800 μl saline. Two hundred microliters of the cell suspension provided total cell counts using the blood cells analysis system (Sysmex KX-21, Japan). Another 400 μl was used for eosinophil counts. These cells were centrifuged and resuspended in 400 μl of solution containing 20 μl of 2% Eosin Y sodium solution (Amresco, high-purity grade, USA), 20 μl acetone

solution, and 360 μl deionized water, in which only the eosinophils are viable. The number of eosinophils was subsequently assessed using a hemocytometer (Sigma, USA) and a microscope (Olympus, Japan).

Lung histology

Following lavage, the left lungs were collected for histology. Samples were incubated in fixing solution (saturated TNT:formalin:glacial acetic acid = 15:5:1) at room temperature for 24 h and then stained with hematoxylin and eosin (H&E, Sigma, USA). Finally, the tissues were embedded in paraffin, sectioned into 10- μm slices, and examined under a microscope (Leica DM 4000B, Germany).

IL-4 and IFN- γ

The pulmonary levels of interleukin (IL)-4 and interferon (IFN)- γ were measured using enzyme-linked immunosorbent assay (ELISA) technique according to the manufacturer's instruction (IL-4 kit: Bender, Germany; IFN- γ kit: eBioscience, USA). The right lungs were removed and a tissue homogenate was prepared (1 g/ml in saline) prior to assessment of IL-4 and IFN- γ by ELISA. The detection limits of IL-4 and IFN- γ were 1.5 and 15 pg/ml, respectively.

Statistical analysis

Results were expressed as mean $\pm$ SE, and one-way analysis of variance (ANOVA) was applied on statistical analysis; p values $< .05$ were considered significant. The dose-effect relationships were plotted with Origin 6.1 software.

Results

Assessment of airway responsiveness

The OVA-treated groups (groups B, C, and D) exhibited greater airway response to MCH compared with saline-treated control group (group A) (Figure 3), and FA exposure significantly enhanced the degree of airway reactivity in OVA-immunized rats in a dose-dependent manner. For the FA-exposed, non-immunized rats, airway responsiveness was higher than in the negative control but lower than the FA-exposed OVA groups (groups C and D) (Figure 3). Additional data analyses (Table 1) were performed by measuring EC25 in a dose-dependent manner for both the Rrs (R-area) and Cldyn, and showed a significant increase in the airway response in FA-exposed rats compared with controls.

Lung histology

Lung histology results demonstrated airway structural changes in OVA-immunized groups (Figure 4) that would be associated with asthma in humans. Gaseous FA exposure

aggravated the asthma-like effects in immunized rats, with the most obvious changes of the airway structure emerging in the OVA + 2500 ppb FA exposure group (Figure 4). Slight but insignificant pulmonary abnormalities were observed in the rats exposed to FA only.

Cell infiltrations in the bronchoalveolar lavage fluid (BALF)

For the FA-exposed groups, both the total cells and eosinophils detected in bronchoalveolar lavage fluid (BALF) were dramatically increased ($p < .01$), when compared with the OVA group and saline group, in a dose-dependent manner. A significant increase ($p < .05$) in eosinophil counts was observed as FA exposure was amplified in OVA-immunized/challenged rats (Figure 5, A and B). The FA-only exposed group (2500 ppb) demonstrated no difference in cell counts when compared with controls. However, the FA-only group had obviously fewer eosinophils compared to the OVA-only exposed group.

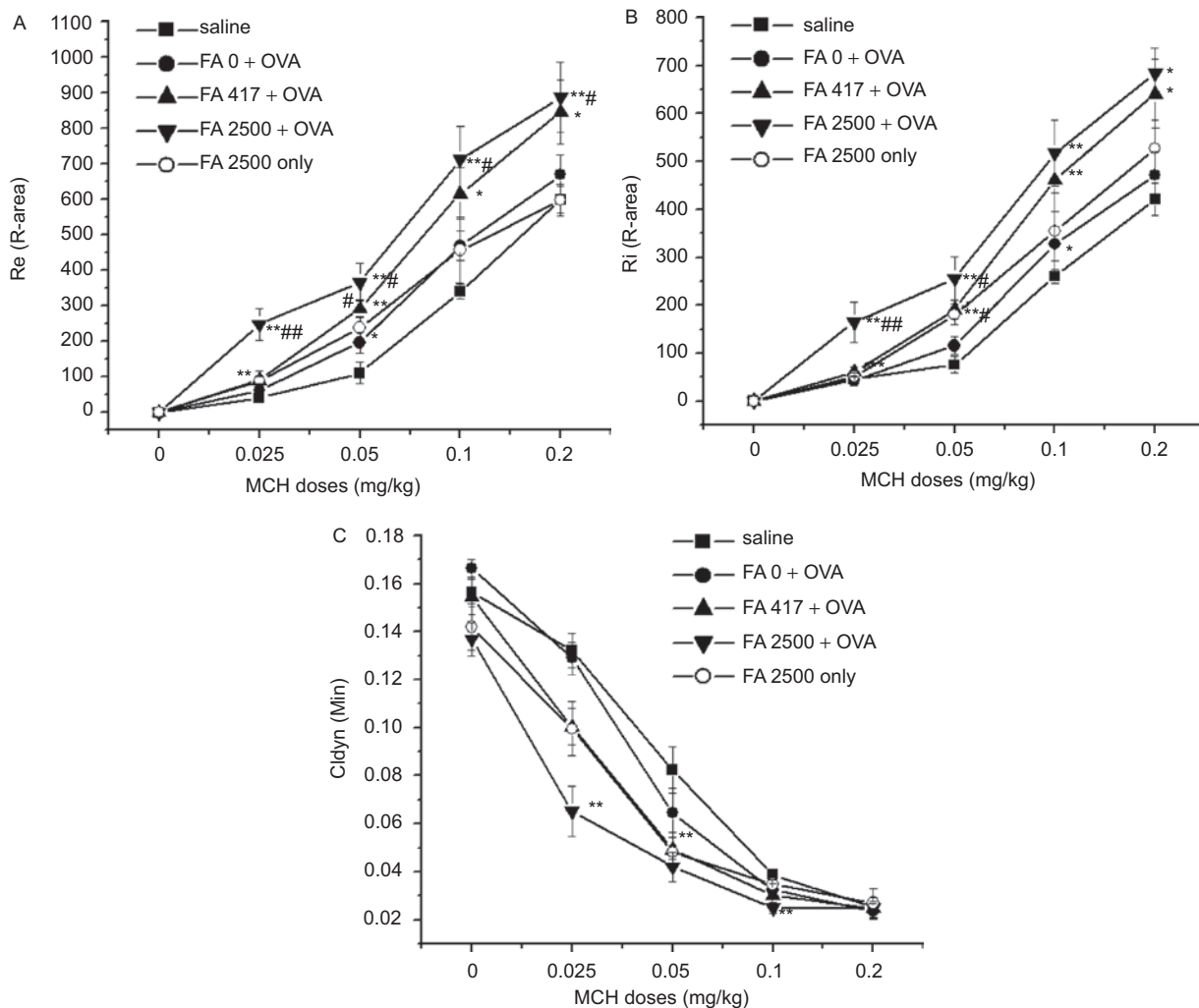


Figure 3. Effects of exposure to FA on airway reactivity to MCH in rats. A: R-area of Re with different dose of MCH; B: R-area of Ri with different dose of MCH; C: the peak value of Cldyn with different dose of MCH. (■: the saline group; ●: the OVA-immunized group; ▲: 417 ppb FA-OVA group; ▼: 2500 ppb-OVA group; ○: 2500 ppb only group. *: $p < 0.05$, **: $p < 0.01$, compared with negative control; #: $p < 0.05$, ##: $p < 0.01$, compared with OVA-immunized group. $n = 8$.)

Table 1. Airway reactivity measurement as EC25 in experimental rats. (*: $p < 0.05$, **: $p < 0.01$, compared with negative control; #: $p < 0.05$, compared with OVA-immunized group. $n = 8$.)

Group	EC25 (mg/kg) (mean $\pm$ SE)		
	Re (R-area)	Ri (R-area)	Cldyn (Min)
A	0.05971 $\pm$ 0.00481	0.0566 $\pm$ 0.00625	0.02962 $\pm$ 0.00561
B	0.04552 $\pm$ 0.00581	0.04817 $\pm$ 0.00539	0.0229 $\pm$ 0.00275
C	0.04011 $\pm$ 0.00246 **	0.04383 $\pm$ 0.00209 *	0.01566 $\pm$ 0.00113 * #
D	0.03129 $\pm$ 0.00586 **	0.03386 $\pm$ 0.005 *	0.0119 $\pm$ 0.00257 * #
E	0.0278 $\pm$ 0.00539 **	0.04383 $\pm$ 0.00857	0.02183 $\pm$ 0.00693

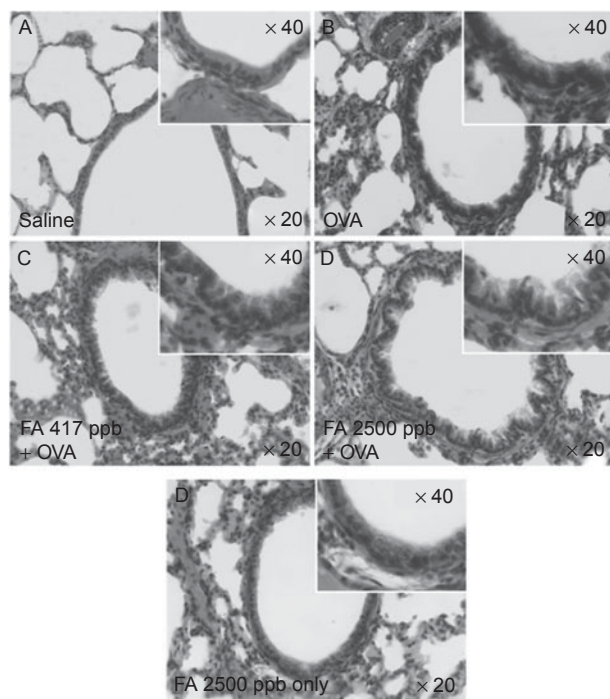


Figure 4. Histological examination of lung tissue airway remodeling (A: the saline group; B: the OVA-immunized group; C: 417 ppb FA-OVA group; D: 2500 ppb FA-OVA group; E: FA 2500 ppb alone group. Lung tissue was fixed, stained with H&E, and sectioned in 10- μ m slices.)

IL-4 and IFN- γ levels

FA exposure enhanced the presence of IL-4 in a dose-dependent manner. However, the FA-only group exhibited much lower levels of IL-4 when compared with the other groups. For IFN- γ , levels in the FA only group were markedly higher (Figure 6, A and B).

Discussion

Establishment of a rat model for FA-induced airway hyperresponsiveness

Airway responsiveness is a recognized indicator for the severity of asthma. For this study, the MCH provocation test as described by Brusasco et al. (2001) was used to document the phenotype. In comparison with techniques that require the removal of an animal's lungs or airways, this has obvious advantages. The mechanical ventilator controlled the breathing patterns and the lungs were analyzed in situ, securing near normal conditions during the

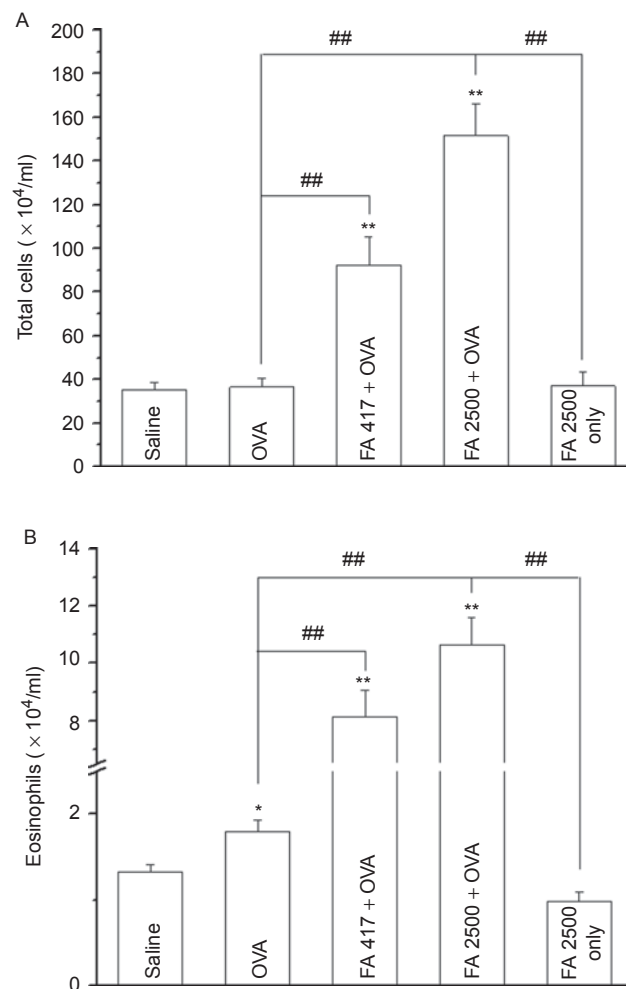


Figure 5. Cell infiltration of the airway in different experimental groups. (A: total cell counts in BALF among experimental groups; B: eosinophils in BALF of different groups. *: $p < 0.05$, **: $p < 0.01$, compared with negative control; #: $p < 0.01$, compared with OVA-immunized group. $n = 8$.)

systemic assessments (Drazen et al., 1999). The R-area of the respiratory resistance (RL, including Re and Ri) and peak values (Min) of Cldyn have been adopted as sensitive indexes (Drazen et al., 1999). The underlying physiological basis of these parameters is based on two factors: RL explains the state of the large airways, whereas changes in Cldyn reflect the variation in small airways or the parenchyma (Drazen et al., 1999; Karol, 1994). The left shift in the dose-response curve indicates a lowered threshold for the airway response to a specific airway stimulus (Gillis et al., 1999; Leigh et al., 2002). Airway remodeling was examined by H&E staining on the lung sections (Boulet et al., 2007). This is believed to be a critical factor in the development of asthma symptoms such as airway hyperresponsiveness and airway obstruction (Elias, 2000; Leigh et al., 2002; Yamauchi et al., 2006).

Differential adjuvant and irritant effect of FA on respiratory systems

The role of FA in the pathogenesis of asthma is obscured by the complex interactions of FA on the respiratory system.

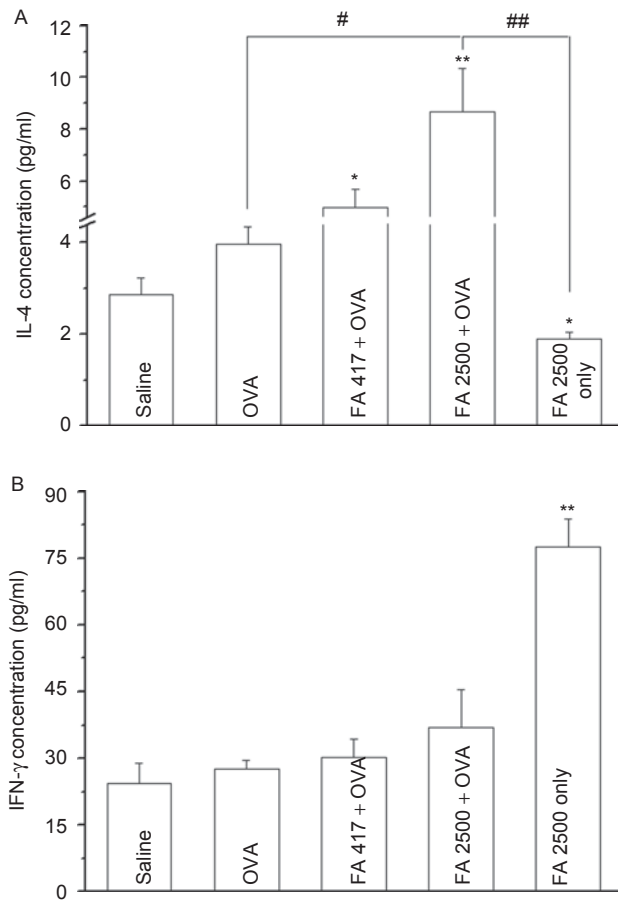


Figure 6. IL-4 and IFN- γ levels in different experimental groups. (A: pulmonary IL-4 levels among different groups; B: pulmonary IFN- γ levels in each group. *: $p < 0.05$, **: $p < 0.01$, compared with negative control; ##: $p < 0.01$, compared with OVA-immunized group. $n = 8$.)

In this study, we therefore analyzed the adjuvant as well as irritant effects of FA by comparing two dosages of FA with OVA groups, FA only and a control group, respectively.

In terms of the adjuvant effect of FA, graduated airway responsiveness to increasing doses of FA was observed (for the OVA-immunized asthma rats, Figure 3). At the highest exposure level of 2500 ppb FA, significantly higher RL and lower Cldyn values were observed compared with other groups, even for those cases in which the lowest dose of MCH (0.025 mg/kg) was applied. This phenomenon was also observed on histological staining (Figure 4). FA exposure exacerbated the degree of airway structural changes in OVA-treated rats, especially in the group of highest FA + OVA exposure (group D). This adjuvant effect of FA was also reported in earlier studies of the immune pathway (Tarkowski et al., 1995; Riedel et al., 1996). Tarkowski's work demonstrated a significant increase in ovalbumin (OVA)-specific IgE titers in mice exposed intranasally to OVA with 1667 ppb of gaseous FA exposure for 10 days. By comparison, low levels of this antibody were produced in mice that were either not exposed at all or only exposed to FA once a week (Tarkowski et al., 1995). Similarly, experiments conducted in guinea pigs showed that short-term exposure to

a low concentration of FA (250 ppb) significantly influenced the presence of asthma (10/12 animals) in comparison with the negative control (3/12 animals), concomitant with increasing production of anti-OVA antibodies (Riedel et al., 1996).

In terms of the irritant effect of FA, the FA-only group showed increased airway responsiveness compared with controls (Figure 3). An earlier experiment in guinea pigs reported that long-term exposure to ≥ 300 ppb FA (8h) or to > 9000 ppb (2h) increased pulmonary resistance and airway responsiveness to acetylcholine (ACH) (Swiecichowski et al., 1993). Lasting airway hyperresponsiveness was noted on the day 8 after moderate FA exposure (day 29) (Figure 1). At the same time, slight but statistically insignificant differences were observed in the airway structure of these animals (Figure 4E). The FA-only group was examined at 8 days post-exposure in order to exclude the potential for acute effects of FA in the animals. Hence, the effects of FA would possibly have been even more apparent if the animals were on the last day of exposure.

To elucidate the inflammatory effects of FA, cytokine levels and the number of eosinophils present in BALF were examined. IL-4 and IFN- γ represent Th2- and Th1-type immune cytokines, respectively, and have been studied in animal models of inflammation associated asthma, e.g., Marsha. (1999). Eosinophils are widely recognized for their association with asthma severity (Hamelmann et al., 2001). Data from this study showed that the IL-4 levels and the number of eosinophils were markedly increased in OVA-immunized rats exposed to FA. In contrast, levels of IFN- γ were not significantly affected under the same conditions. These results suggest a prominent Th-2 type reaction in FA-exposed, OVA-immunized groups, which is consistent with earlier results showing increased levels of IL-4 in BALF and serum IgE in FA-exposed mice (Lu et al., 2005; Qiao et al., 2007). FA exposure alone significantly stimulated IFN- γ secretion as much as threefold when compared with control rats (Figure 6). By contrast, IL-4 levels were reduced, indicating a prominent Th-1 type reaction stimulated by gaseous FA exposure. Eosinophil counts revealed no significant difference (Figure 5). In this case, we suspected that gaseous FA itself may act as an irritant on the airways and induce airway hyperresponsiveness (Douwes et al., 2002), which may be involved in the pathogenesis of non-eosinophilic asthma.

Taken together, we hypothesized that both pathways may participate in the development of FA-induced asthma or asthma-like symptoms. For sensitization, the effect of the combined exposure occurs even with low levels of FA, whereas the inflammatory effects have only been shown with high levels of FA exposure. In the presence of common allergens, FA may increase the sensitization rate by boosting the Th-2 type reaction and cell activation. FA alone may also act as an irritant inducing airway hyperresponsiveness and obstruction, mimicking other low MW chemicals (Molfino et al., 1991; Riedel et al., 1988; Larsen et al., 2001).

Applications of FA in a rat model of allergen-induced inflammation and airway hyperresponsiveness

Two main parameters, FA exposure and allergen administration, were explored in this study. OVA was selected as a common allergen that has been widely used in animal models (Kurucz et al., 2006; Kumar et al., 2008); to induce sensitization and inflammatory responses in rats simultaneously, experimental rats were treated with gaseous FA before and during the process of OVA immunization. Further work will be necessary in order to bring the model closer to particles and gases encountered by people in the home environment, such as airborne exposures to house dust mite and cockroach allergens.

In this study, FA exposure was withheld for 7 days to avoid the acute irritant effect of FA on the respiratory tracts of the animals. In parallel, the 2500 ppb FA-only group exposure was conducted. Using this approach, the dual effects of FA were demonstrated.

A dynamic-consistent FA exposure strategy described by Li et al. (2003) was adopted using a small environmental chamber to generate preset concentrations of gaseous FA from 10% formalin reagent (purity >99.99%). Therefore, stable and pure gaseous FA could be generated throughout the experiment (Figure 2B), and the relative humidity was maintained at $45 \pm 5.0\%$, facilitating FA exposure through the airways. Because FA exposure conditions (e.g., the air relative humidity and temperature) (Mi et al., 2006) influence the FA deposition pattern in vivo, failure to control the FA exposure might cause outcome discrepancies between studies and thus might lead to erroneous conclusions.

The experimental model at hand offers us an in vivo system to further determine the effect of FA on asthma and possibly other respiratory diseases where low molecular weight chemicals may play a role. The current lack of information regarding the underlying mechanisms of chemical-induced asthma is likely due to a lack of sufficient animal models. Therefore, the establishment of an FA-induced hyperresponsiveness model is of potentially great value, and will provide a basis for further study of the role of low-molecular-weight chemicals (e.g., isocyanate) in asthma induction.

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