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Naphthoquinone enhances antigen-related airway inflammation in mice

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Running head title: NQ on allergic airway inflammation

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ABSTRACT

We have previously demonstrated that diesel exhaust particles (DEP) enhance antigen-related airway inflammation in mice (Takano et al., 1997). Further, our recent study has shown that organic chemicals in DEP, rather than their carbonaceous nuclei, are important contributors to the aggravating effects on the airway inflammation (Yanagisawa et al., 2006). However, the components in DEP responsible for the enhancing effects on the model remain to be identified. We investigated the effects of naphthoquinone (NQ), one of extractable chemical compounds of DEP, on antigen-related airway inflammation, local expression of cytokine proteins, and antigen-specific Igs production in mice. Pulmonary exposure to NQ dose-dependently aggravated antigen-related airway inflammation characterized by infiltration of eosinophils and lymphocytes around the airways and an increase in goblet cells in the bronchial epithelium. Combined exposure to NQ and antigen enhanced the local expression of interleukin (IL)-4, IL-5, eotaxin, macrophage chemoattractant protein-1, and keratinocyte chemoattractant as compared with exposure to antigen or NQ alone. Also, NQ exhibited adjuvant activity for the antigen-specific production of IgG_1 and IgG_{2a}. These results provide the first experimental evidence that NQ can enhance antigen-related airway inflammation *in vivo*, and NQ can partly play a role in the pathogenesis of DEP toxicity on the condition.

Key Words: naphthoquinone, antigen, airway inflammation, immunogloblin

Introduction

Diesel exhaust particles (DEP) are major contributors to the atmospheric particulate air pollution in metropolitan areas. DEP have correlated to lung cancer, pulmonary fibrosis, chronic alveolitis [1], and edematous changes [2]. Also, DEP have been implicated to modulate allergic reactions [3, 4]. DEP enhance the antigen-specific IgE response [5, 6] and aggravate airway inflammation induced by repetitive intratracheal instillation of antigen *in vivo* [7-10].

DEP are complicated particles consisting of carbonaceous nuclei and a vast number of organic chemical compounds such as polyaromatic hydrocarbons, aliphatic hydrocarbons, heterocycles, and quinones. Previous studies have indicated that organic chemicals extracted from DEP result in induction of apoptosis [11], increase oxidative stress [12], and induce inflammatory cells [13-15] through the release of proinflammatory molecules [15, 16] *in vitro*. Also, we have recently demonstrated that extracted organic chemicals from DEP rather than residual carbonaceous nuclei of DEP after extraction, predominantly enhance antigen-related airway inflammation in mice [17]. However, detailed researches about the component(s) of DEP responsible for their effects on the respiratory system and/or pulmonary diseases remain unsatisfied especially *in vivo*.

A variety of quinones have been identified as DEP components [18, 19]. Quinones themselves have toxicological properties to serve as alkylating agents and to interact with, for example, flavoproteins to generate reactive oxygen species (ROS), which can induce biological injury [20-23]. Phenanthraquinone (PQ) is one of quinones involved in DEP [19, 23]. We have recently shown that PQ induces recruitment of

inflammatory cells such as eosinophils and neutrophils into the lung with the lung expression of proinflammatory molecules such as interleukin (IL)-5 and eotaxin *in vivo* [24]. More recently, we have demonstrated that PQ aggravates antigen-related airway inflammation in mice [25]. PQ has also adjuvant activity for antigen-specific Igs [25]. These studies suggest that quinones may be key compounds in the enhancing effects of DEP on allergic airway diseases.

Naphthoquinone (NQ) is another extractable compound from DEP [25]. NQ contributes to DEP toxicity through the reduction of superoxide dismutase activity *in vitro* [26], which raises the possibility that NQ may facilitate airway inflammatory conditions *in vivo*. The aim of the present study was to elucidate the effects of NQ on airway inflammation, the local expression of cytokines such as IL-4 and IL-5 and chemokines such as eotaxin, macrophage chemoattractant protein (MCP)-1, and keratinocyte chemoattractant (KC) and Igs production related to antigen exposure.

Methods

Animals

Male ICR mice 6 to 7 wk of age and weighing 29 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. They were fed a commercial diet (Japan Clea Co.) and given water ad libitum. Mice were housed in an animal facility that was maintained at 24 to 26□ with 55 to 75% humidity and a 12-h light/dark cycle. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. All animal studies were approved by the Institutional Review Board.

Study protocol

Mice were divided into eight groups (fig. 1). The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) once a week for 6 wk. The ovalbumin (OVA) group received 1μg of OVA (Sigma Chemical, St. Louis, MO) dissolved in the same vehicle bi-weekly for 6 wk. The NQ group received NQ at a dose of 1.58 ng/animal, 15.8 ng/animal, or 158 ng/animal dissolved in the same vehicle every week for 6 wk. The NQ + OVA group received the combined treatment in the same protocol as the NQ and the OVA groups. In each group, vehicle, NQ, OVA, or OVA + NQ was dissolved in 0.1-ml aliquots and inoculated by the intratracheal route through a polyethylene tube under anesthesia with 4% halothane (Hoechst, Japan, Tokyo, Japan) as previously described [8, 27]. The animals were studied 24 h after the last intratracheal administration.

Blood retrieval and analysis

Mice were anesthetized with diethyl ether. The chest and abdominal walls were opened, and blood was retrieved by cardiac puncture. Plasma was prepared and frozen at – 80□ until assayed for antigen-specific IgG_1 and IgG_{2a}.

Bronchoalveolar lavage (BAL)

The trachea was cannulated after the collection of blood. The lungs were lavaged with 1.2 ml of sterile saline at 37□, instilled bilaterally by syringe. The lavage fluid was harvested by gentle aspiration. This procedure was conducted two more times.

The average volume retrieved was 90 % of the 3.6 ml that was instilled; the amounts did not differ by treatment. The fluid collections were combined and cooled to 4□. The lavage fluid was centrifuged at 300 g for 10 min, and the total cell count was determined on a fresh fluid specimen using a hemocytometer. Differential cell counts were assessed on cytologic preparations. Slides were prepared using Autosmear (Sakura Seiki Co., Tokyo, Japan) and were stained with Diff-Quik (International reagents Co., Kobe, Japan). A total of 500 cells were counted under oil immersion microscopy (n = 7-8 in each group).

Histologic evaluation of leukocyte accumulation and goblet cell metaplasia in the lung

After the collection of blood, the lungs were fixed by intratracheal instillation of 10% neutral phosphate-buffered formalin at a pressure of 20 cm H_2O. After separation of the lobe, 2-mm-thick blocks were taken for paraffin embedding. Sections 3 μm thick were stained with H & E to observe and evaluate the degree of infiltration of eosinophils or neutrophils + mononuclear cells around the all airways. The sections were stained with periodic acid Schiff (PAS) to evaluate the degree of proliferation of goblet cells in the bronchial epithelium. Histological analyses were performed using a microscope. The degree of eosinophil or neutrophil + mononuclear cell infiltration around the airways and the proliferation of goblet cells in the bronchial epithelium were graded in a blind fashion: 0 = not present; 1 = very slight; 2 = slight; 3 = moderate; 4 = moderate to marked; 5 = marked. '1' was defined as an inflammatory reaction affecting less than 20% of the airways (less than 20% goblet cells stained with PAS); '2': 20-40% of the airways were affected; '3': 40-60%; '4': 60-80%, and '5': more than 80% of the airways were affected (n = 4-5 in each group) [10].

Quantitation of cytokine and chemokine protein levels in the lung

In a separate series of experiments, the animals were exsanguinated and the lungs were subsequently homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma, St Louis MO), 0.1 mM phenylmethanesulphonyl fluoride (Nacalai Tesque, Kyoto, Japan), 1μM pepstatin A (Peptide Institute, Osaka, Japan) and 2μM leupeptin (Peptide Institute) as described previously [8]. The homogenates were then centrifuged at 105,000 g for 1 h. The supernatants were stored at -80□. Enzyme-linked immunosobent assays (ELISA) for IL-4 (Amersham, Buckinghamshire, UK), IL-5 (Endogen, Cambridge, MA), interferon (IFN)-γ(R&D systems, Minneapolis, MN), eotaxin (R&D systems), MCP-1 (R&D systems), and KC (R&D systems) in the lung tissue supernatants were conducted using matching antibody pairs according to the manufacture's instruction. The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the readings at 450 nm were converted to pg/ml using values obtained from standard curves generated with the limits of detection of 5 pg/ml, 5 pg/ml, 5 pg/ml, 3 pg/ml, 1.5 pg/ml, and 2pg/ml for IL-4, IL-5, IFN-γ, eotaxin, MCP-1, and KC, respectively (n = 7-8 in each group).

Antigen-specific IgG determination

Antigen-specific IgG_1 or IgG_{2a} antibodies were measured by ELISA with solid-phase antigen [8, 10]. In brief, microplate wells (Dynatech, Chantilly, VA) were coated with OVA overnight at 4□ and then incubated at room temperature for 1 h with PBS containing 1% bovine serum albumin (BSA; Sigma) containing 0.01% thimerosal (Nakalai Tesque). After washing, diluted samples were introduced to the microplate and

incubated at room temperature for 1 h. After another washing, the wells were incubated at room temperature for 1 h with biotinylated rabbit anti-mouse IgG_1 or IgG_{2a} (Zymed Laboratories, San Francisco, CA). After another washing, the wells were incubated with horseradish-peroxidase-conjugated streptavidin (Sigma) at room temperature for 1 h. The wells were then washed and incubated with o-phenylenediamine and H_2O_2 in dark at room temperature for 30 min. The enzyme reaction was stopped with 4 N H_2SO_4. Absorbance was read at 492 nm. Each plate incubated a previously screened standard plasma that contained a high titer of anti-OVA antibodies. The results were expressed in titers, calculated based on the titers of the standard plasma. Cut off values for antibody-positive plasma were set to hold as the mean value of absorbance of preimmune plasma (n = 7-8 in each group).

Statistical analysis

Data were reported as mean ± SEM. Differences among groups were analyzed by ANOVA followed by *Fisher's* PLSD test (Stat view version 4.0; Abacus Concepts, Inc, Berkeley, CA). Significance was assigned to P values smaller than 0.05.

Results

NQ accelerates antigen-related airway inflammation

To evaluate the effects of NQ on allergic airway inflammation, we investigated the cellular profile of BAL fluid and lung histology in eight groups of mice 24 h after the last intratracheal instillation.

There was a tendency that the number of neutrophils in BAL fluid was greater in the NQ or the OVA group than in the vehicle group (*) (fig. 2a). The number was greater in the NQ + OVA groups than in the OVA ($P < 0.05$ versus the NQ (158ng) + OVA group) or NQ ($P < 0.01$ versus the NQ (158 ng) + OVA group) group. The number of macrophages was greater in the NQ groups than in the vehicle group ($P < 0.05$ for the NQ (15.8ng) group: fig. 2b). There was a tendency that the number of eosinophils was greater in the OVA group than in the vehicle group (*) (fig. 2c). The number was significantly greater in the NQ (158 ng) + OVA group than in the OVA ($P < 0.01$) or the NQ ($P < 0.05$) group. There was a tendency that the number of mononuclear cells was greater in the NQ (15.8ng) or the OVA group than in the vehicle group (*) (fig. 2d). The number was further greater in the NQ + OVA groups than in the OVA group ($P < 0.05$ versus the NQ (158 ng) + OVA group) or in the NQ groups (N. S.).

To quantitate the infiltration of inflammatory cells around the airways, we estimated the magnitude of the histopathological changes in the lung specimens stained with H & E (fig. 3). Instillation of 15.8ng of NQ increased the number of infiltrated eosinophils as compared with vehicle instillation. Instillation of OVA increased the numbers as compared with vehicle instillation (fig. 3a and fig. 4). Combined treatment with NQ and OVA significantly increased the number as compared with OVA treatment alone ($P < 0.05$ versus the NQ (1.58 ng) + OVA group, $P < 0.01$ versus the NQ (15.8 ng) + OVA group, or the NQ (158 ng) + OVA group) or NQ treatment alone ($P < 0.05$ versus the NQ (1.58 ng) + OVA group, $P < 0.01$ versus the NQ (15.8 ng) + OVA group or the NQ (158 ng) + OVA group). Instillation of NQ showed negligible increases in the numbers of infiltrated neutrophils and mononuclear cells as compared with vehicle instillation. There was a tendency that instillation of OVA increased the numbers as compared with vehicle instillation (*) (fig. 3b and Fig. 4). The NQ + OVA groups

showed significant increases in the number as compared with the OVA group ($P < 0.01$ versus the NQ (1.58 ng) + OVA group, the NQ (15.8 ng) + OVA group, or the NQ (158 ng) + OVA group) or the NQ groups ($P < 0.01$ versus the NQ (1.58 ng) + OVA group, the NQ (15.8 ng) + OVA group, or the NQ (158 ng) + OVA group).

Effects of NQ on the goblet cell metaplasia in the airways after antigen challenge

To evaluate bronchial epithelial injury and hypersecretion of mucus, lung sections were stained with PAS. There was a tendency of that the number of goblet cells was slightly greater in the NQ (15.8 ng) group or in the OVA group than in the vehicle group ((*) fig. 3c and fig. 5). The combined treatment with NQ and OVA significantly increased the number as compared with the OVA ($P < 0.01$) or the NQ ($P < 0.05$ versus the NQ (15.8 ng) + OVA group, $P < 0.01$ versus the NQ (158 ng) + OVA group) treatment.

Effects of NQ on local expression of cytokines in the presence of antigen

To explore the role of local expression of Th2 cytokines such as IL-4 and IL-5 or that of a Th1 cytokine, IFN-γ, in the enhancing effects of NQ on antigen-related airway inflammation, we quantitated the protein levels in the lung tissue supernatants 24 h after the last intratracheal instillation (table 1). Protein level of IL-4 was significantly greater in the NQ (158 ng) ($P < 0.05$), the OVA, and the NQ (158 ng) + OVA ($P < 0.01$) groups than in the vehicle group. The level was greater in the NQ (158 ng) + OVA group than in the NQ (158 ng) ($P < 0.05$). However, the level was not significantly different between the OVA and the NQ (158 ng) + OVA groups. Protein levels of IL-5 and IFN-γ in the NQ (158 ng) group were comparable to those in the vehicle group. There was a

tendency that OVA exposure increased the levels of IL-5 as compared with vehicle exposure. The level of IL-5 was greater in the NQ (158 ng) + OVA group than in the NQ (158 ng) group ($P < 0.01$). IFN-γ level was greater in the NQ (158 ng) + OVA group than in the vehicle (N. S.), the NQ (N. S.), or the OVA ($P < 0.05$) group.

Effects of NQ on local expression of chemokines in the presence of antigen

To explore the role of local expression of chemokines such as eotaxin, MCP-1, and KC in the enhancing effects of NQ on antigen-related airway inflammation, we quantitated the protein levels of the chemokines in the lung tissue supernatants 24 h after the last intratracheal instillation (table 1). These protein levels in the NQ (158 ng) group were comparable to those in the vehicle group. OVA exposure increased the levels of the chemokines as compared with vehicle exposure ($P < 0.05$ for MCP-1). The level of eotaxin was greater in the NQ (158 ng) + OVA group than in the NQ (158 ng) ($P < 0.05$) or the OVA group (N. S.). The levels of MCP-1 and KC were greater in the NQ (158 ng) + OVA group than in the NQ (158 ng) ($P < 0.01$ for MCP-1, $P < 0.05$ for KC) group or the OVA ($P < 0.05$) group.

NQ has adjuvant activity for antigen-specific production of IgG_1 and IgG_{2a}

To examine whether NQ has adjuvant activity for antigen-specific Igs production, we measured antigen-specific IgG_1 (fig. 6) and IgG_{2a} (fig. 7) 24 h after the last intratracheal instillation. There was a tendency that the titer of antigen-specific IgG_1 was greater in the OVA group than in the vehicle group (*). The combination of NQ plus OVA, in particular at its dose of 1.58 ng/animal, increased antigen-specific production of IgG_1 as compared with OVA alone ($P < 0.05$ for NQ (1.58 ng) + OVA).

There was a tendency that the titer of antigen-specific IgG_{2a} was greater in the OVA group than in the vehicle group. The combination of NQ plus OVA, in particular at its dose of 158 ng/animal, increased antigen-specific production of IgG_{2a} as compared with vehicle, NQ, or OVA alone ($P < 0.05$ for NQ (158 ng) + OVA).

Discussion

The present study has demonstrated that NQ administered by intratracheal route deteriorates antigen-related airway inflammation in mice, which is characterized by the infiltration of inflammatory leukocytes in both the bronchoalveolar spaces and the lung parenchyma. NQ also exaggerates antigen-related goblet cell metaplasia. The enhancing effects are concomitant with the increased lung expression of IL-5, eotaxin, especially with that of MCP-1, and KC. Also, NQ exhibits adjuvant activity for the antigen-specific production of IgG_1 and IgG_{2a}.

We have previously shown that intratracheal administration of DEP enhances airway inflammation related to antigen [8]. DEP consist of carbonaceous nuclei and a vast number of organic chemical compounds such as polyaromatic hydrocarbons, aliphatic hydrocarbons, heterocycles, and quinones [26, 28, 29]. Previous *in vitro* studies have indicated that exposure of macrophages to organic chemicals extracted from DEP increases the oxidative stress-inducible protein heme oxygenase-1 [12] and results in induction of apoptosis [11]. Organic chemicals in DEP can also affect inflammatory effector leukocytes [13-15], and trigger the release of proinflammatory molecules such as IL-1, IL-8, tumor necrosis factor -α, and regulated on activation and normal T cells expressed and secreted *in vitro* [15, 16]. We have recently shown that intratracheal instillation of organic chemicals in DEP enhances the neutrophilic lung inflammation

related to endotoxin *in vivo* [27]. Furthermore, our more recent study has shown that organic chemicals in DEP, rather than their carbonaceous nuclei, predominantly enhance antigen-related airway inflammation in mice [17]. However, the responsible organic chemicals in DEP have not been fully identified.

Quinones are involved in DEP [18, 19]. Quinones posses toxicological properties to serve as alkylating agents and to interact with, for example, flavoproteins to generate ROS, which can induce biological injury [20-23]. We have recently shown that single intratracheal administration of PQ, one of quinones involved in DEP [19, 23], can induce recruitment of inflammatory cells such as eosinophils and neutrophils with the local expression of proinflammatory molecules such as IL-5 and eotaxin *in vivo* [24]. Our study has demonstrated that pulmonary exposure to PQ enhances antigen-related airway inflammation *in vivo* [25]. On the other hand, NQ, another extractable chemical compound in DEP, generates free radical, binds to thiol containing proteins, and irreversibly inactivates them [26]. In the present study, the numbers of eosinophils, neutrophils, and mononuclear cells in the BAL fluid and in the lung tissues were greater in the NQ + OVA groups than in the OVA group in a dose dependent manner with overall trend. The results indicate that NQ can exacerbate antigen-related airway inflammation. Furthermore, in our previous study [25], PQ has not shown significant aggravating effects on the magnitude of airway inflammation and goblet cell metaplasia in the lung histology. In the present study, combined treatment with NQ and antigen histologically exacerbated airway inflammation and goblet cell metaplasia as compared with treatment with antigen alone. The previous study [25] and the present results suggest that NQ, rather than PQ, may be an important quinone involved in the enhancing effects of DEP on antigen-related airway inflammation.

Allergic asthma is often associated with predominant local expression of Th2 type cytokines including IL-4 and IL-5. Among chemokines, eotaxin is essential for eosinophil recruitment in antigen-related airway inflammation [30, 31]. In fact, our previous studies have confirmed that the exaggerated allergic airway inflammation induced by DEP parallel the local elevation of the inflammatory proteins [7, 8]. MCP-1 has a chemoattractant effect of $CD4^+$ and $CD8^+$ T lymphocytes [32]. MCP-1 also plays a role in recruitment of eosinophils to inflammatory sites [33]. KC induces airway inflammation with mucus hypersecretion [34-36]. KC plays a role also in airway hyperresponsiveness [36]. Therefore, we measured local expression of the cytokines and chemokines in the present study. NQ further enhanced the lung expression of these proteins, especially those of MCP-1 and KC, which were elevated by the antigen challenge alone. Thus, the results suggest that NQ aggravates antigen-related airway inflammation, at least in part, via the enhancement of the local expression of these proteins. Interestingly, in our previous study, PQ did not significantly enhance the expression of these proteins in the presence of antigen ([25] and unpublished observation). The results may also indicate that the enhancing effects of NQ on antigen-related airway inflammation may be stronger than those of PQ. It is also possible that the enhancing effects of NQ are mediated through different pathways from those of PQ. Future studies are needed to clarify the mechanisms of the deteriorated airway inflammation induced by the quininones.

IgG with antigen is a strong agonist for eosinophil degradation *in vitro* [37]. Furthermore, late asthmatic reactions are associated with IgG antibody [38]. We have reported that DEP enhance antigen-specific production of IgG induced by OVA *in vivo* [7, 8]. In the present study, the combined intratracheal administration of NQ and antigen induced a greater increase in both OVA-specific IgG_1 than the other administration. It is

likely that NQ also plays a role in the enhancing effects of DEP in view of antigen-specific Ig production. The magnitude of inflammatory response after treatment with NQ appeared to be directly correlated with dose, with the highest response at 158 ng, however, OVA-specific IgG_1 was not the case. A bell-shaped dose-response was observed with the highest immune response at a much lower dose (1.58 ng) followed by a decline with increasing doses. The 100-fold difference in optimal dose might be explained by a shift towards other IgG-isotypes at higher doses. Indeed, OVA-specific IgG_{2a} titer, a hallmark of Th1-biased immune response, was highest in the NQ (158 ng) + OVA group, although this titer did not show apparent dose-response curve. Also, there was a tendency that IFN-γ, representative Th1 cytokine, was higher in the NQ (158 ng) + OVA group than in the OVA group. These results indicate that NQ enhances Th2-related IgG_1 production at lower dose, whereas it increases Th1-related IgG_{2a} production at higher doses. On the other hand, titers of IgE, another critical Ig in the hallmark of asthma, were not significantly different between the experimental groups (data not shown). Significant production of antigen-specific IgE in the antigen-sensitized and challenged group as compared to the vehicle group has been found at 9 wk or later in our previous experimental protocol [8]. Thus, another experimental protocol may be needed in future to elucidate the effects of NQ on adjuvant activity for antigen-specific IgE production. Collectively, the detailed impacts of NQ on humoral responses and their mechanisms await further studies.

In other concept, there is a correlation between airway inflammation and airway responsiveness [39]. Further, goblet cell metaplasia is strongly related to the airway pathophysiology [40]. In the present study, we observed that NQ enhanced airway inflammation with goblet cell metaplasia related to repetitive antigen exposure,

suggesting that NQ may facilitate airway hyperresponsiveness on the condition. We are conducting further experiments to study the effects of NQ on airway hyperrreactivity.

In conclusion, the present study has shown that NQ can enhance allergic airway inflammation. The enhancing effects are concomitant with the increased lung expression of IL-4, IL-5, eotaxin, especially with that of MCP-1, and KC. These results suggest that environmental quinones are implicated in the increasing prevalence of allergic asthma in recent years and may play, at least in part, a role in the DEP-toxicity against allergic diseases.

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Figure Legend

Fig. 1.- Experimental design.

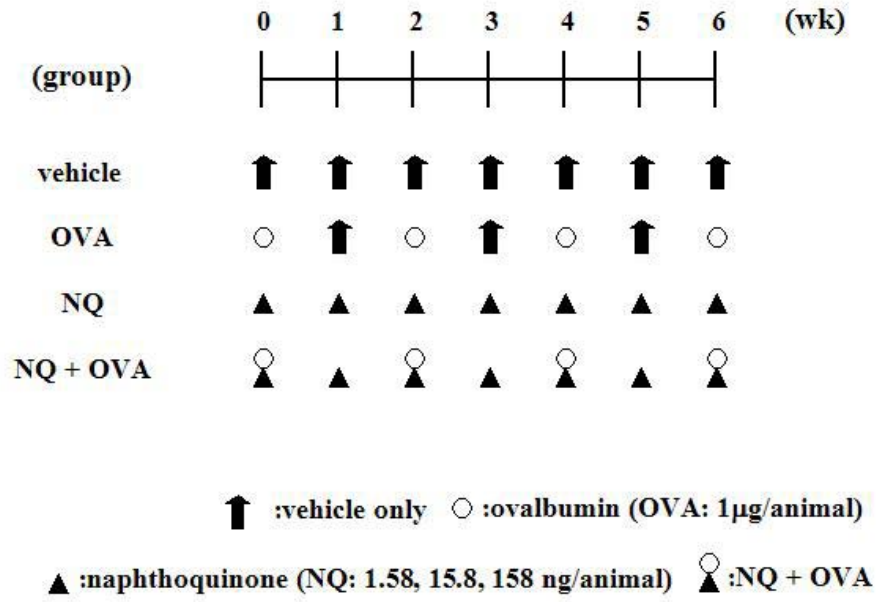

Fig. 2.- Cellular profile (a: neutrophils, b: macrophages, c: eosinophils, d: mononuclear cells) in bronchoalveolar lavage (BAL) fluid. Eight groups of mice were intratracheally administered vehicle, three doses of naphthoquinone (NQ), ovalbumin (OVA), or a combination of NQ + OVA for 6 wk. BAL was conducted 24 h after the last intratracheal instillation. Differential cell counts were assessed with Diff-Quik staining. Results are presented as mean ± SEM (n = 7-8 in each group). * $P < 0.05$ versus vehicle. ** $P < 0.01$ versus vehicle. † $P < 0.05$ versus NQ. †† $P < 0.01$ versus NQ. $^{\#}$ $P < 0.05$ versus OVA. $^{\#\#}$ $P < 0.01$ versus OVA.

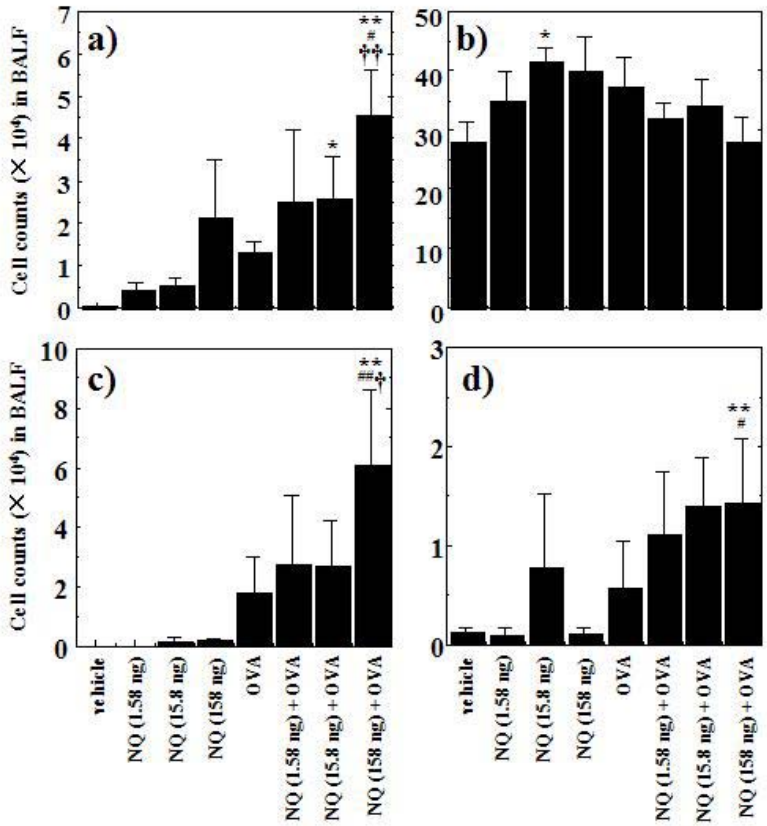

Fig. 3.- Degree of infiltration of inflammatory cells around the airways and goblet cells in the bronchial epithelium. Animals received intratracheal instillation of vehicle, three doses of NQ, OVA, or OVA + NQ for 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration. Sections were stained with H & E for measurement of inflammatory cells around the airways or with PAS for goblet cells in the bronchial epithelium. Degree of infiltration of eosinophils (a), neutrophils + mononuclear cells (b), and goblet cells (c) was estimated. Values are mean ± SEM (n = 4-5 in each group). * $P < 0.05$ versus vehicle. ** $P < 0.01$ versus vehicle. † $P < 0.05$ versus NQ. †† $P < 0.01$ versus NQ. $^{\#}$ $P < 0.05$ versus OVA. $^{\#\#}$ $P < 0.01$ versus OVA.

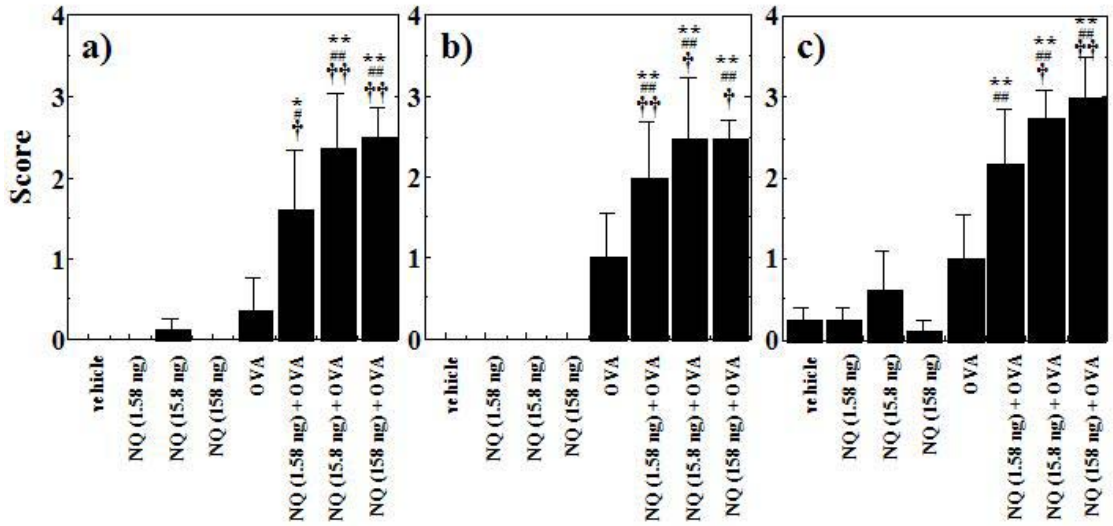

Fig. 4.- Representative histological findings of the H & E-stained lung obtained from the (a) vehicle, (b) NQ (158 ng), (c) OVA, (d) NQ + OVA (158 ng) group. Animals received intratracheal instillation of vehicle, NQ, OVA, or NQ + OVA for 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration. Original magnification ×25.

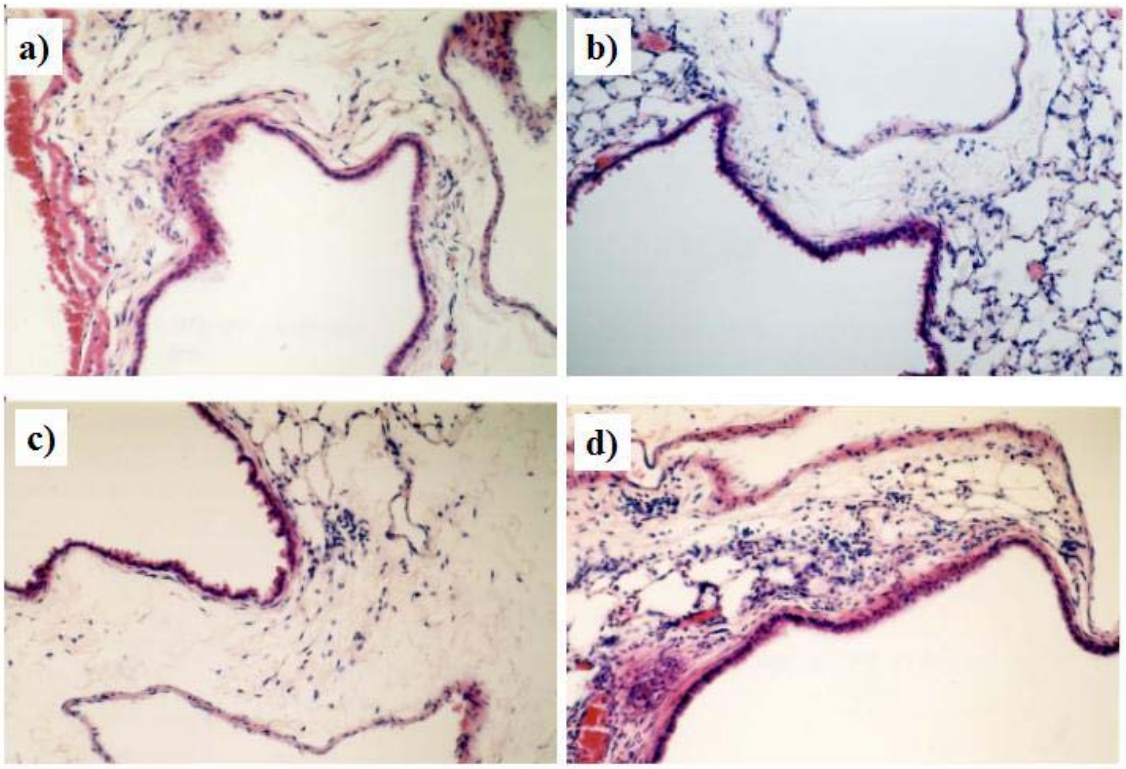

Fig. 5.- Representative histological findings of the periodic acid Schiff (PAS)-stained lung obtained from the (a) vehicle, (b) NQ (158 ng), (c) OVA, (d) NQ + OVA (158 ng) group. Animals received intratracheal instillation of vehicle, NQ, OVA, or NQ + OVA for 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration. Original magnification ×50.

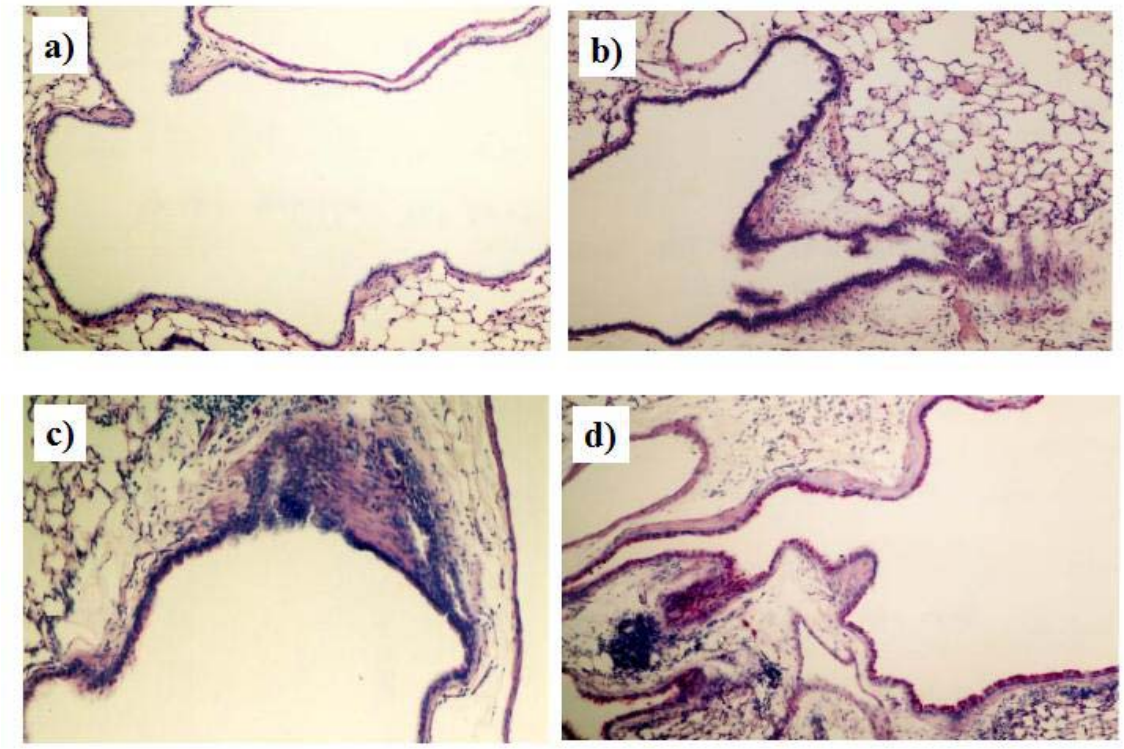

Fig. 6.- Antigen-specific IgG_1 titers. Eight groups of mice were intratracheally administered vehicle, OVA, three doses of NQ, or the combination of NQ and OVA for 6 wk. Plasma samples were retrieved 24 h after the last intratracheal instillation. Antigen-specific IgG_1 was analyzed using enzyme-linked immunosorbent assay. Results are expressed as mean ± SEM (n = 7-8 in each group). * $P < 0.01$ versus vehicle. † $P < 0.01$ versus NQ. $^{\#}$ $P < 0.05$ versus OVA.

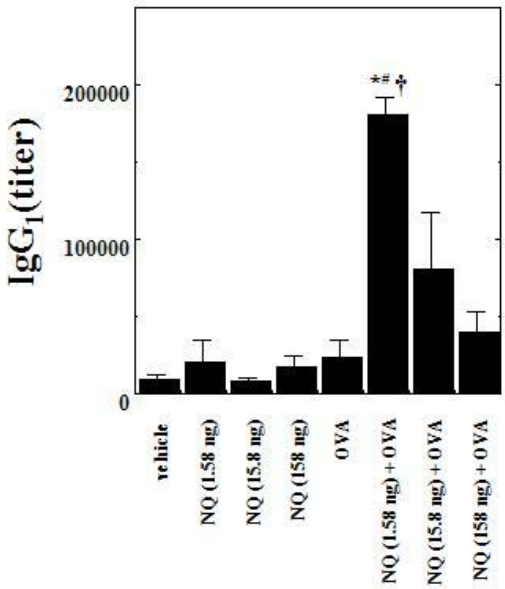

Fig. 7.- Antigen-specific IgG_{2a} titers. Eight groups of mice were intratracheally administered vehicle, OVA, three doses of NQ, or the combination of NQ and OVA for 6 wk. Plasma samples were retrieved 24 h after the last intratracheal instillation. Antigen-specific IgG_{2a} was analyzed using enzyme-linked immunosorbent assay. Results are expressed as mean ± SEM (n = 7-8 in each group). * $P < 0.05$ versus vehicle. † $P < 0.05$ versus NQ. $^{\#}$ $P < 0.05$ versus OVA.

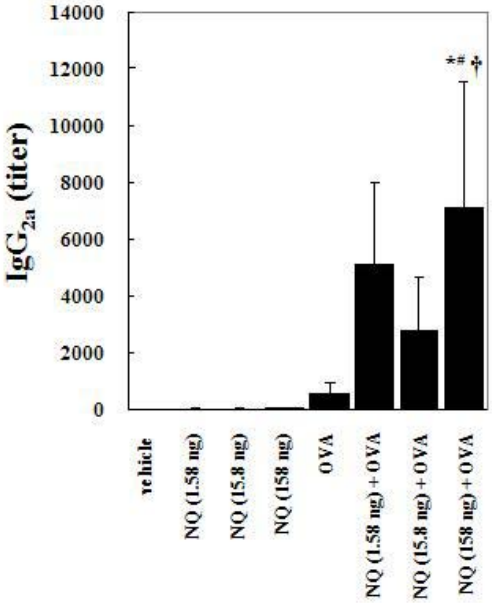

Table 1.- Protein levels of cytokines in the lung.

Group	n	IL-4	IL-5	IFN-γ
		pg/total lung supernatants		
vehicle	8	0 ± 0	14.56 ± 1.42	1264.1 ± 33.1
NQ (158 ng)	8	2.30 ± 0.37 *	10.38 ± 1.21	1250.0 ± 41.4
OVA	8	4.06 ± 0.82 **	46.08 ± 15.98	1223.3 ± 83.4
NQ (158 ng) + OVA	7	4.50 ± 0.89 ** †	77.04 ± 29.94 ** ††	1380.3 ± 43.6 #

Four groups of mice were intratracheally inoculated with vehicle, naphthoquinone (NQ), ovalbumin (OVA), or the combination of NQ + OVA for 6 wk. Lungs were removed and frozen 24 h after the last intratracheal administration. Protein levels in the lung

tissue supernatants were analyzed using enzyme-linked immunosorbent assays. Results are shown as mean ± SEM. * $P < 0.05$ versus vehicle. ** $P < 0.01$ versus vehicle. † $P < 0.05$ versus NQ (158). †† $P < 0.01$ versus NQ (158). # $P < 0.05$ versus OVA.

Table 2.- Protein levels of chemokines in the lung.

Group	(n)	eotaxin	MCP-1	KC
		pg/total lung supernatants		
vehicle	8	66.31 ± 2.52	18.44 ± 4.05	17.90 ± 4.61
NQ (158 ng)	8	74.76 ± 3.50	21.14 ± 3.72	19.81 ± 6.74
OVA	8	233.70 ± 90.78	44.64 ± 9.37 *	32.38 ± 10.46
NQ (158 ng) + OVA	7	405.97 ± 181.02 ** †	79.71 ± 17.59 ** †† #	106.49 ± 40.51 ** † #

Four groups of mice were intratracheally inoculated with vehicle, naphthoquinone (NQ), ovalbumin (OVA), or the combination of NQ + OVA for 6 wk. Lungs were removed and frozen 24 h after the last intratracheal administration. Protein levels in the lung

tissue supernatants were analyzed using enzyme-linked immunosorbent assays. Results are shown as mean ± SEM. * $P < 0.05$ versus vehicle. ** $P < 0.01$ versus vehicle. † $P < 0.05$ versus NQ (158). †† $P < 0.01$ versus NQ (158). # $P < 0.05$ versus OVA.