

Neutrophil infiltration and release of IL-8 in airway mucosa from subjects with grain dust-induced occupational asthma

H.-S. PARK, K.-S. JUNG*, S.-C. HWANG†, D.-H. NAHM and H.-E. YIM‡

Departments of Allergy and Clinical Immunology, †Pulmonary and Critical Care Medicine and ‡Pathology, Ajou University School of Medicine, Suwon and *Department of Internal Medicine, Hallym University School of Medicine, Seoul, Korea

Summary

Background The immuno-pathological mechanism for occupational asthma induced by grain dust (GD) remains to be clarified. There have been few reports suggesting the involvement of neutrophils inducing bronchoconstriction after inhalation of GD.

Objective To further understand the role of neutrophil in the pathogenesis of GD-induced asthma.

Materials and methods We studied the phenotype of leucocytes of the bronchial mucosa in patients with GD-induced asthma. Bronchial biopsy specimens were obtained by fiberoptic bronchoscopy from six subjects with GD-induced asthma. Six allergic asthma patients sensitive to house dust mite were enrolled as controls. Bronchial biopsy specimens were examined by immunohistochemistry with a panel of monoclonal antibodies to tryptase-containing mast cell (AA1), activated eosinophil (EG2), pan T-lymphocyte (CD3) and neutrophil elastase (NE). Induced sputum was collected before and after the GD-bronchoprovocation test. The IL-8 level in the sputum was measured using ELISA.

Results There was a significant increase in the number of AA1⁺ and NE⁺ cells in bronchial mucosa of GD-induced asthma, compared with those of allergic asthma ($P=0.01$, $P=0.01$, respectively). No significant differences were observed in the number of EG2⁺ and CD3⁺ cells ($P=0.13$, $P=0.15$, respectively). IL-8 was abundant in the sputum of all GD-induced asthma patients and significantly increased after the bronchial challenges compared with the baseline value ($P=0.03$).

Conclusion These findings support the view that neutrophil recruitment together with mast cells may contribute to the bronchoconstriction induced by GD. A possible involvement of IL-8 was suggested.

Keywords: neutrophil, grain dust-induced occupational asthma, IL-8, induced sputum

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Introduction

Chronic inhalation of grain dust (GD) has been shown to cause acute and chronic airway injury characterized by bronchitis and airflow obstruction [1–4]. Longitudinal studies have shown accelerated deterioration of pulmonary function in these grain workers [5], the severity of which appears to be related to the concentration of airborne GD in the work environment [4,6]. The presence of specific IgE

antibodies in individuals who have clinical lower respiratory symptoms with exposure to GD, has been demonstrated in up to 40% of symptomatic workers in the same workplace [7].

Airway inflammation is clearly an important characteristic of asthma. Inhalation of GD causes an inflammatory response characterized by neutrophil recruitment to the lower respiratory tract. *In vitro* studies indicate that extracts of GD are capable of recruiting neutrophils by several mechanisms; both endotoxin- and non-endotoxin-induced chemotaxis, activation of complement, and release of alveolar macrophage-derived neutrophil chemotactic activity [8].

Correspondence: Dr H.-S. Park, Department of Allergy and Clinical Immunology, Ajou University School of Medicine, Paldalgw Wonchondong San-5, Suwon, Korea.

IL-8 has been recognized as an inflammatory cytokine with strong potency in neutrophil activation and chemotaxis [9,10].

In order to improve understanding of the role of neutrophil in GD-induced occupational asthma, we performed immunohistochemical analysis of bronchial mucosa using monoclonal antibodies to inflammatory cells including neutrophil, T lymphocyte, mast cell and eosinophil, and looked at the changes of IL-8 level in the induced sputum before and after the GD inhalation challenges.

Materials and methods

Subjects

All of the 43 subjects participating in this study had been exposed to grain dust composed of corn, rye, wheat and barley while working for the Dongbang Animal Feed Industry in Suwon, Korea. Among them, 15 (34.9%) had complained of work-related respiratory symptoms. When bronchoprovocation test with grain dust was performed on 15 symptomatic workers, six were confirmed to have occupational asthma based upon significant bronchoconstrictions on GD-bronchoprovocation test, clinical history and work-related symptoms (group I). Three of them had serum specific IgE antibodies to GD which were detected by ELISA and all had specific IgG antibody. Six allergic asthma patients sensitive to house dust mite, never exposed to GD were enrolled as controls (group II). Their clinical characteristics are summarized in Table 1. None of the subjects had taken any antiasthmatic medication including corticosteroid for 8 weeks prior to the study. The subjects gave their written informed consent as regulated by Ajou University Hospital, Suwon, Korea.

Preparation of extracts

GD was obtained from the subjects' workplace. It was extracted with phosphate buffered saline [(PBS, pH 7.5), L5w/v] at 40°C for 1 h followed by centrifugation at 5000 rpm. The supernatants were passed through the syringe filter (MIS, USA:0.25 µm pore-sized) and used for the bronchoprovocation tests at a concentration of 1:10 w/v. Some of the supernatant was dialysed (the cut-off molecular weight was 6000 Da) against 4 L of distilled water at 40°C for 48 h, and lyophilized at -70°C for the preparation of antigens and used in ELISA for detection of serum specific IgE antibody.

Protocol

All six subjects who had complained of work-related respiratory symptoms when exposed to GD in the workplace

were admitted to the Allergy Clinic at Ajou University Hospital, and before the inhalation challenge, a standard protocol was followed in all cases. A screening evaluation including a complete history and physical examination, pulmonary function tests including methacholine bronchial challenge test, chest X-ray, and electrocardiogram were performed upon admission. A peripheral blood sample was obtained for total and differential counts of leucocytes. Baseline forced expiratory flow were performed using a MultiSPIRO SX/PC (Multi SPIRO Inc, USA) with standard protocols.

Bronchoprovocation test with grain dust

Airway responsiveness to methacholine was tested as previously described elsewhere with some modifications [11]. Bronchoprovocation tests were performed according to standard studies of occupational asthma [7,12]. On the first day, normal saline was administered from a nebulizer 646 connected to a dosimeter (DeVilbiss Co., USA) operated at a pressure of 20 psi. The subject was asked to inhale the aerosol five times to the vital capacity, and then was observed for 7 h. A sputum induction test was done and collected sputum was used as a baseline sample. On a different day, GD extracts were inhaled from 1:1000 to 1:10 w/v until FEV₁ declined by 20%. In the pulmonary function test, forced vital capacity, forced expiratory volume in one second (FEV₁), and forced expiratory flow rate (FEF₂₅₋₇₅) were measured with MultiSPIRO SX/PC (MultiSPIRO Inc, USA) before and 10 min after each inhalation. Then, FEV₁ and FEF₂₅₋₇₅ were measured every 10 min during the first hour, and then hourly for 7 h after the challenge. And then, a second sputum induction test was performed and collected sputum was used as a postchallenge sample. A positive reaction was defined as a decrease in FEV₁ of more than 20% from the baseline value.

ELISA for specific IgE antibodies to grain dust extract

The presence of specific IgE antibody to GD was determined by ELISA according to the previously described method [7,12]. Microtitre plates (Dynatech, USA) were first coated with 50 µL/well of GD (5 µg/well) extract, and left at 40°C overnight. Each well was washed three times with 0.05% Tween-phosphate buffered saline (PBST), and the remaining binding sites were blocked by incubation with 350 µL of 3% BSA-PBST for 1 h at room temperature. The wells were then incubated for 2 h at room temperature with 50 µL of either the patients' sera (undiluted) or control sera from 27 patients who showed negative skin-prick test responses to common inhaled allergens as well as GD. After washing three times with PBST, 50 µL of the 1:1000 v/v biotin-labelled goat antihuman IgE antibody

(Vector Co., USA) was added to the wells and incubated for 2 h at room temperature. The wells were then washed three times with PBST and incubated with 1:1000 v/v streptavidin-peroxidase (Sigma Co. USA) for 30 min before another washing step which was followed by incubation with 100 μ L 2 N sodium azide. The absorbance was read at 410 nm by an automated microplate reader. All assays were performed in triplicate. The cut-off absorbance of positive IgE binding was determined as 0.064, which was derived from the mean and two standard deviations of the absorbance value of the control subjects.

Induction of sputum methodology

Sputum was induced using a previously described method [13,14]. Immediately before the sputum expectoration, each subject was pretreated with 200 μ g salbutamol, administered by means of a metered dose inhaler. The subjects then inhaled nebulized sterile 3% saline solution for 20 min through an ultrasonic nebulizer (Omron Co., Japan). Sputum sample was collected throughout the procedure. After being instructed to wash the mouth, spit out the saliva and blow the nose, the subjects were asked to cough and expectorate sputum into a clean plastic container.

Sputum processing

Collected sputum and saliva samples were immediately processed as previously reported [14]. The volumes of the induced sputum were determined, and equal volumes of phosphate buffered saline (PBST) were added. The samples were then mixed by vortex mixer for 1 min and centrifuged for 20 min at 3000 rpm. The supernatants were aspirated and frozen at -200°C .

ELISA for IL-8 in the induced sputum

To evaluate the role of IL-8, the induced sputum from all six subjects with GD asthma were collected before the challenge as baseline value and at 7 h after the GD bronchial challenge test. The IL-8 level was measured using ELISA kit (R&D system, USA) under the manufacturer's guidance. The albumin content within the sputum was measured by nephelometry and the IL-8 level was presented as $\mu\text{g}/\text{albumin content}$.

Bronchoscope and bronchial biopsies

Fibreoptic bronchoscopy was performed after the inhalation challenge with GD as described previously [15]. At least two endobronchial biopsies were taken through the bronchoscope (Olympus BF, type IT 200; Olympus Co., Tokyo, Japan) with sterile forceps (FB 15C; Olympus Co.)

from the bronchial mucosa of the right intermediate bronchus. The biopsy specimen was fixed in 4% formaldehyde/1% glutaraldehyde in 0.1 M PBS, then dehydrated and embedded in paraffin for immunohistochemistry.

Immunohistochemistry and quantification

Sections (4 μm thick) were obtained from biopsy specimens using microtome (Shandon, HM340I, Japan). Activation markers of inflammatory cells were assessed by immunohistochemistry on paraffin-embedded sections using a panel of inflammatory cells to monoclonal antibodies: anti-CD3 (Dako, Denmark), antibody to secretory form of eosinophil cationic protein (EG2, Pharmacia, Sweden), antineutrophil elastase (NE, Dako, Denmark), and antibody to mast cell tryptase (AA1, Southampton University, UK). The mouse monoclonal antibody to mast cell tryptase was kindly provided by Andrew Walls from Southampton University, which had been previously characterized for their specificity and reactivity. Biotin-conjugated rabbit antimouse antibodies (1:200 in TBS, Sigma, USA) were applied as secondary antibody and detected with the streptavidin-biotin complex using LSAB kit (Dako, CA, USA) and aminoethyl carbazole as chromogen. They were counterstained with Mayer's haematoxylin. The numbers of positively stained cells were counted in four fields per one tissue by superimposing a grid of 100 points (intersection of crosses covering a surface area of 0.5 mm^2) under an eyepiece graticulate at a magnification of $\times 200$, and expressed as the mean number of cells per mm^2 as reported by Fokkens *et al.* [16]. For negative controls, the mouse IgG, antibodies (1:200 v/v) were incubated with omission of primary antibodies. To avoid observer bias, the slides were coded before analysis and read blind.

Statistical analysis

The Mann-Whitney *U* and Wilcoxon-signed rank tests were applied using the SPSS version 7.0 (Chicago, USA) to evaluate the statistical differences between the data: a *P*-value of less than 0.05 was regarded as significant.

Results

Bronchoprovocation test

Table 2 summarized the changes of FEV_1 during the asthmatic response. Five subjects of group I showed early asthmatic responses. One showed a dual asthmatic response. The inhaled dose of GD antigen to induce 20% fall of FEV_1 was described in Table 2.

Inflammatory cell infiltrate in bronchial mucosa

Figure 1 shows AA1⁺ (1a) and NE⁺ cell (1b) in the bronchial mucosa from the subjects with GD-induced asthma. Figure 2 demonstrates the comparison of individual inflammatory cell counts expressing AA1, CD3, EG2 and NE cell markers between GD-induced asthma and allergic asthma patients. All subjects had both AA1⁺ and EG2⁺ cells. The numbers of AA1⁺ and NE⁺ cells were significantly higher in GD-induced asthma than those of allergic asthma ($P=0.01$, $P=0.01$, respectively). No significant differences were observed in the numbers of EG2⁺ and CD3⁺ cells ($P=0.13$, $P=0.15$, respectively).

IL-8 level in the induced sputum

Table 2 shows changes of IL-8/albumin level in the induced

sputum before and after GD- bronchoprovocation test. IL-8 was abundantly present in the baseline value of induced sputum from all subjects with GD-induced asthma and it significantly increased after the challenge ($P=0.03$), in contrast to no significant changes in those working in the same workplace, but without occupational asthma ($P>0.05$, data not shown).

Discussion

Several mechanisms may be involved in pathogenesis of GD-induced occupational asthma. Our previous study [7] demonstrated the serum specific IgE antibodies and IgE-binding components within GD in six subjects with GD-induced asthma, and suggested that the IgE-mediated reaction is likely to be responsible for their asthmatic symptoms. The failure to detect specific IgE antibodies to GD in the sera of three workers with positive challenges might indicate the involvement of other immunological or non-immunological mechanism in the induction of their asthmatic symptoms. Although there has been speculation that IgG may have a role in the pathogenesis of asthma [17], our previous study on the effect of anti-IgG4 antibody on basophil histamine release from six challenge-proven GD-asthma patients, suggested that the possibility of specific IgG4 as a sensitizing antibody seemed to be very low [12].

Recently, neutrophil involvement in the pathogenesis of occupational asthma has been suggested. Neutrophilia had been noted in bronchoalveolar lavage fluid in subjects with late asthmatic reaction induced by isocyanate challenge test [18]. Inhalation of grain sorghum dust in normal volunteers could induce an increased peripheral neutrophil count and chemotactic response to GD [19]. *In vitro* study revealed the release of substances presenting neutrophil chemotactic activity by bronchial epithelial cell in response to GD [20]. So far, the mechanism of neutrophil chemotaxis and the function of the neutrophils in GD-induced asthma are not well documented. In this study, the numbers of NE⁺ cells were significantly higher in the bronchial mucosa of GD-induced asthma than in those of allergic asthma. Our previous study dealing with neutrophil chemotactic activity of GD-asthma [15] revealed that serum neutrophil chemotactic activity significantly increased at 30 min after GD inhalation challenge relative to the baseline value, and then significantly decreased at 240 min, whereas no significant changes of neutrophil chemotactic activity occurred in asthmatic patients with negative result on grain dust-bronchoprovocation test. These findings suggest possible involvement of neutrophils as an effector cell in the development of GD-induced bronchoconstriction in exposed workers.

IL-8 was recognized as a key chemokine to induce neutrophil recruitment and activation. Early evidence for

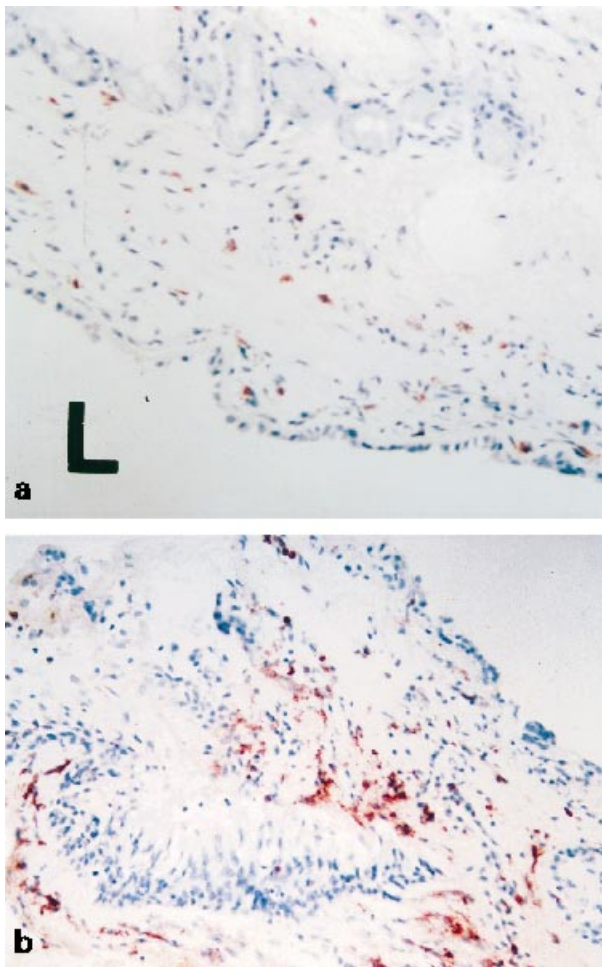


Fig. 1. Localization of tryptase-containing mast cell (a) and elastase-containing neutrophils (b) in the bronchial biopsy from subjects with grain dust-induced occupational asthma. Magnification in a & b $\times 200$.

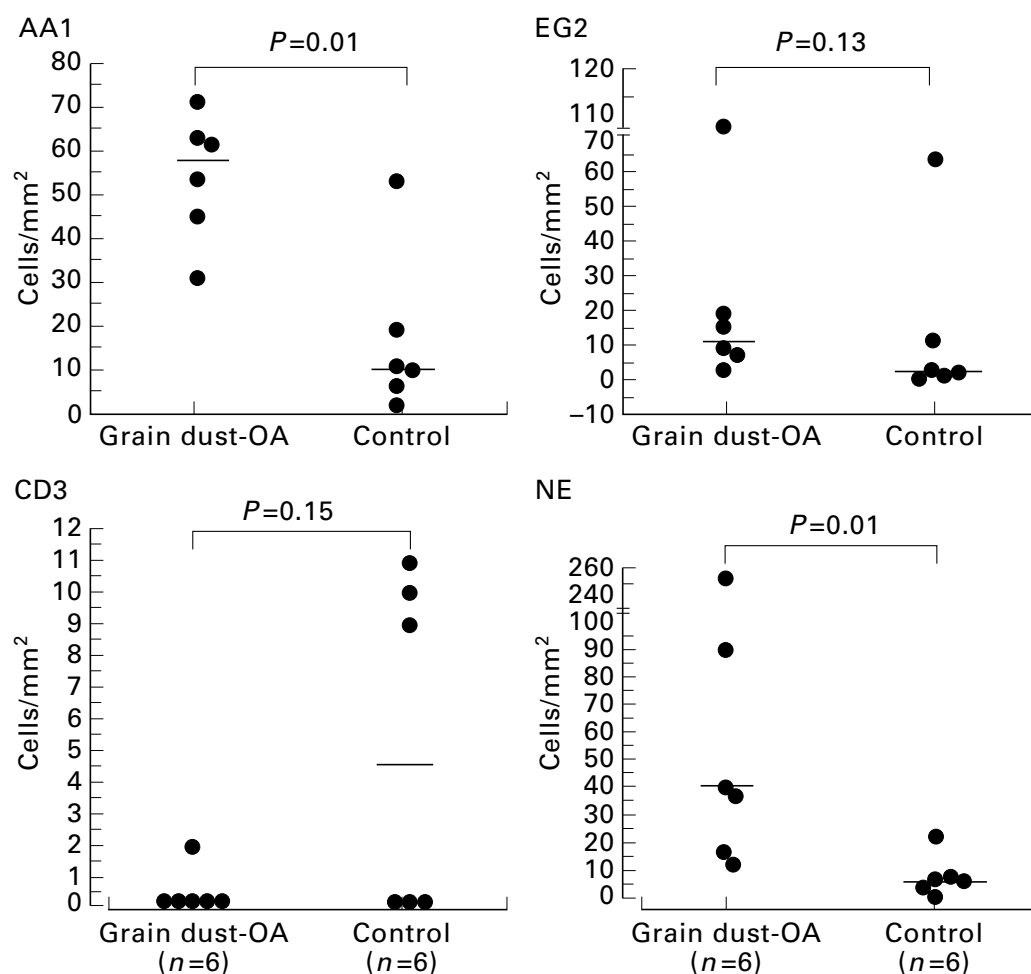


Fig. 2. Individual cell count expressing AA1 (mast cells), EG2 (activated eosinophils), CD3 (pan T cells) and NE (neutrophils) in bronchial mucosa of grain dust-induced occupational asthma and allergic asthma as controls, expressed as the number of positive cells per mm². Median values are represented by horizontal bars.

the involvement of this chemokine in allergic inflammation was obtained from a study which demonstrated increased expression of IL-8 immunoreactivity in bronchial biopsies of asthmatic patients when compared with biopsies from normal subjects [21]. Release of IL-8 had also been reported to be increased in the bronchoalveolar large (BAL) fluid of asthmatic patients [22] and in the nasal secretions of atopic patients in response to nasal allergen challenge [23]. However, neutrophils have not only been known as merely terminal effector cells; they have also been known to affect other cells by releasing various cytokines [9, 24–26]. Recently, it has been reported that mast cells are able to release IL-4, TNF α , IL-8, leukotrienes and platelet activating factors which could induce neutrophil chemotaxis [27,28]. In the present study, the numbers of tryptase-secreting mast cells and neutrophils were significantly higher in GD-induced asthma than in allergic asthma. The

IL-8 level in the induced sputum significantly increased after the bronchial challenge test compared with the baseline level in contrary to no significant change in asthmatic subjects showing a negative result on GD-bronchoprovocation test. These results suggest that activated neutrophils associated with mast cell may participate in the development of GD-induced bronchoconstriction in exposed workers, and IL-8 released after exposure to GD might contribute to neutrophil infiltration into the airway mucosa. Further investigations will be needed to elucidate the exact mechanism of IL-8 released after exposure to GD.

Induction of sputum production by inhalation of hypertonic saline was widely used to investigate the bronchial constituents of airway secretions in asthmatic patients. The results obtained from them are consistent and could yield more concentrated secretions than those obtained from bronchoscopy [29–32]. Biochemical analysis of the fluid

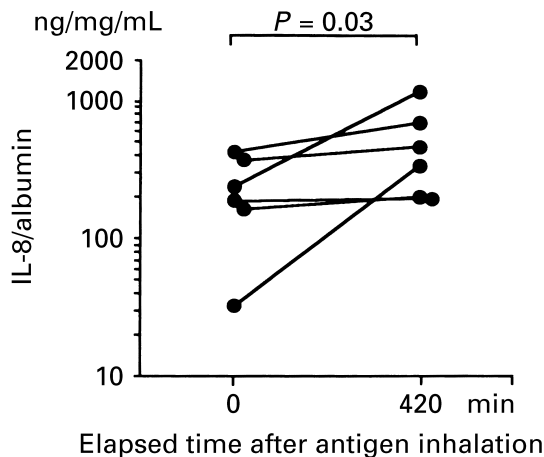


Fig. 3. Release of IL-8 in the induced sputum before and after the bronchial challenge in six grain dust-asthma patients showing a positive result on the bronchial challenge test. A significant increase of IL-8 relative to the baseline value was noted after the bronchial challenge test ($p = 0.03$).

phase of the induced sputum samples should take into consideration the variable dilutions by hypertonic saline and saliva [13]. In this study, we measured albumin in both saliva and sputum from the same subjects. The albumin level in saliva was much lower than those in induced sample in the same subjects, which suggested that sputum IL-8 and albumin level presented in this study was derived from the subglottic airway. Therefore, in order to correct the dilution factor and IL-8 level in the induced sputum was presented as ratio to albumin (IL-8/albumin).

In conclusion, these findings support the view that both neutrophils and mast cells are involved in the development of GD-induced asthma and that IL-8 may contribute to neutrophil recruitment.

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