

Neutrophil activation following TDI bronchial challenges to the airway secretion from subjects with TDI-induced asthma

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Summary

Background The immunopathological mechanism for occupational asthma induced by toluene diisocyanate (TDI) remains to be further clarified. There have been few reports suggesting involvement of neutrophils in inducing bronchoconstriction after TDI inhalation.

Objectives To further understand the role of neutrophils in the pathogenesis of TDI-induced asthma.

Materials and methods Eight TDI-induced asthmatic subjects were classified as group I, and five exposed workers who had complained of work-related symptoms and worked in the same workplace, but showed negative bronchial challenges were enrolled as controls (group II). Serum neutrophil chemotactic activity during TDI bronchial challenge test was measured by the Boyden chamber method. Induced sputum was collected before and after the TDI bronchial challenge test. The myeloperoxidase (MPO) and interleukin (IL) -8 levels in the sputum were measured using RIA and ELISA.

Result Serum neutrophil chemotactic activity significantly increased at 10 min ($P = 0.01$), then decreased at 60 min ($P = 0.02$) and remained unchanged for up to 420 min ($P = 0.07$) in group I subjects, while no significant changes were found in group II subjects ($P > 0.05$). MPO and IL-8 were abundantly present in the sputum of all the TDI-induced asthmatic subjects and they increased significantly at 420 min after the bronchial challenges ($P = 0.02$, $P = 0.03$, respectively), while no significant changes were noted in group II subjects ($P > 0.05$).

Conclusion These findings support the view that activated neutrophils may contribute to bronchoconstriction induced by TDI which may be associated with IL-8 release.

Keywords: induced sputum, interleukin-8, neutrophil, TDI-induced occupational asthma

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Introduction

Isocyanates are low-molecular-weight chemicals used in the manufacture of polyurethane forms, varnishes, paints, and plastics. These chemicals are currently the most common cause of occupational asthma in Korea [1] as well as in North America [2].

Although considerable controversy remains regarding the pathogenesis of TDI-induced asthma, several groups of

investigators have identified serum-specific IgE antibodies in sensitized workers: the positive rate varies from 0 to 50% [3–5]. There has been evidence suggesting cell-mediated immunity is involved in which activated lymphocytes similar to allergic asthma, interact with other inflammatory cells such as activated eosinophil and mast cells [6–8]. With regard to the role of the neutrophil, neutrophilia in bronchoalveolar lavage fluid [9] were reported especially among late asthmatic responders with TDI-induced asthma. The recent investigation of bronchial mucosa applying the immunohistochemical method has revealed that neutrophil count is significantly higher in subjects with TDI-induced

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asthma than those of allergic asthma [10]. There has been one previous study showing an increase in serum neutrophil chemotactic activity at 1 h after bronchial challenge test in TDI-induced asthma [11]. To the best of our knowledge, there have been very few reports on neutrophil activation status in trachea-bronchial secretion, and on changes in serum neutrophil chemotactic activity during the TDI bronchial challenge test.

In this study, to further understand the role of neutrophils in the pathogenesis of TDI-induced asthma, we looked at the changes of serum neutrophil chemotactic activity, as well as the changes in MPO and IL (interleukin) -8 levels in induced sputa during the TDI bronchial challenge, and compared them with the positive and negative responders on the TDI bronchial challenge.

Methods

Subjects

Eight subjects with TDI-induced asthma were classified as group I. Five subjects, who had worked in the same workplace with exposure to TDI and complained of lower respiratory symptoms, but showed negative response on TDI bronchial challenge test, were enrolled as controls (group II). Their clinical characteristics of group I and II were summarized in Table 1. One subject with TDI-induced asthma was defined as atopic, as determined by a positive skin test to at least one common allergen extract, but this symptom was not related to exposure to such allergens. All

subjects were treated with inhaled/oral bronchodilators whenever symptoms developed. They underwent an interview, chest radiography, ECG, skin prick test with common allergen extracts, lung function measurement, and inhalation challenge with both methacholine and TDI (Aldrich, Milwaukee, WI, USA).

Bronchial challenge test with methacholine and TDI

The methacholine bronchial challenge test was done according to the method described previously [12]. Briefly, aerosols were generated by a DeVilbiss 646 nebulizer connected to a DeVilbiss dosimeter driven by compressed air (DeVilbiss Co., Doylestown, PA, USA). Five inhalations of normal saline at 5-min intervals were taken followed by a series of successively doubled doses of methacholine (0.075–25 mg/mL) until a 20% fall in FEV₁ was observed. FEV₁ was measured 5 min after the beginning of each set of inhalations of aerosolized methacholine. The methacholine PC₂₀ level was determined by interpolation from the dose-response curve. The TDI bronchial challenge test was performed according to the previously described method [13]. Briefly, the subjects were exposed to TDI (80 : 20 = 2,4 form: 2,6 form, Aldrich) through tidal breathing in a small closed room for 5–15 min until asthmatic symptoms were induced. The concentration of TDI measured by TLD-1, a toxic gas detector with Cheakey (HDA Scientific, Lincolnshire, IL, USA), was 20 p.p.b. FEV₁ and FEF_{25–75%} were measured with a spirometer (MultiSPIRO SX/PC, Tempe, AZ, USA) immediately before exposure

Table 1. Clinical features of the study subjects

Patient	Sex	Age (years)	Atopy	Exposure period (years)	Methacholine PC ₂₀ (mg/mL)	BPT Response	Serum-specific IgE antibody to TDI
Group I							
1	M	42	—	15	1.21	Early	+
2	F	35	—	5	2.4	Early	—
3	M	48	—	10	0.62	Late only	—
4	F	42	—	10	2.0	Dual	—
5	F	38	—	10	1.2	Dual	—
6	F	42	+	7	0.28	Atypical	+
7	F	41	—	5	9.5	Early	—
8	F	50	—	8	4.8	Early	+
Group II							
1	M	28	+	2	> 25	Negative	—
2	M	35	—	3	10.0	Negative	—
3	F	48	+	8	5.0	Negative	—
4	M	29	—	4	4.6	Negative	—
5	M	32	—	3	12.0	Negative	—

BPT, TDI bronchoprovocation test result. +, present. —, absent

and every hour for 8 h after the exposure. Every subject gave a written informed consent as regulated by Ajou University Hospital, Suwon, Korea.

Measurement of neutrophil chemotactic activity

Sera were collected from group I and group II subjects before and 10, 60 and 420 min after the TDI bronchial challenges. Serum neutrophil chemotactic activity was measured using Boyden chamber method with some modifications [14,15]. Neutrophils were obtained from heparinized whole blood of AB-type normal volunteers by sedimentation in 6% dextran dextrose solution followed by centrifugation on Ficoll-Hypaque solution (specific gravity of 1.077) and the supernatants were aspirated and frozen at -20°C hypotonic lysis. Cells containing more than 95% neutrophils were suspended in Hank's balanced salt solution (HBSS) with 0.4% of bovine serum albumin (BSA) at the concentration of 1×10^6 cells/mL. Polycarbonate filter with 5- μm pore size (Osmonics, Livermore, CA, USA) was topped over chemotaxin followed by distribution of neutrophil suspension. The chamber was incubated for 90 min at 37°C in a humidified incubator containing 5% CO_2 . Thereafter, the filter was removed, fixed in 100% methanol, and subsequently stained with Diff Quick stain solution. The number of neutrophils which migrated through the filter was determined microscopically at $40\times$ objective. Five fields were counted per well and the experiments were conducted in quadruplicate. The results were expressed as the mean of the number of migrated neutrophils per field in each sample. Opsonized serum was used in the positive control and HBSS with BSA in the negative control.

Induction of sputum methodology

Sputum was induced using a previously described method [16] and collected before (0 min) and after completing the TDI bronchial challenge test (420 min after the TDI exposure). Immediately before the sputum expectoration, each subject was pretreated with 200 μg 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., Tokyo, Japan). Sputum samples were collected throughout the procedure. After being instructed to spit out saliva in their mouths and to blow their noses, 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 reported previously [16]. The volumes of induced sputum were determined, and equal volumes of

phosphate-buffered saline–0.5% Tween (PBST) were added. The samples were then mixed by vortex mixer for 1 min, centrifuged for 20 min at 3000 r.p.m., and kept frozen at -20°C .

ELISA for IL-8 and MPO in induced sputum

To observe neutrophil activation status, MPO and IL-8 levels were measured in induced sputum from eight subjects with TDI-induced asthma. Sputum was collected twice before the challenge as baseline value, and at 7 h after the TDI bronchial challenge test. MPO level was measured by radioimmunoassay (Pharmacia, Uppsala, Sweden), and IL-8 level with ELISA kit (R & D Systems, Minneapolis, MN, USA). Both assays were performed according to the manufacturer's guidelines. The albumin content within the sputum was measured by nephelometry, and the MPO and IL-8 levels were presented as ratios to the albumin content.

Statistical analysis

The Wilcoxon-signed rank tests were applied using SPSS version 7.0 (Chicago, IL, USA) to evaluate the statistical differences between the data. A *P*-value of 0.05 or less was regarded as significant.

Results

Changes in serum neutrophil chemotactic activity during the TDI bronchial challenge

Sera from groups I and II were collected at 0 (before the challenge test), 30 and 60 min after the TDI bronchial challenge test. Figure 1 shows the changes of serum neutrophil chemotactic activity in group I subjects. All subjects showed similar changing patterns: serum neutrophil chemotactic activity significantly increased ($P = 0.03$) and peaked at 30 min after the TDI inhalation challenges, and thereafter decreased at 60 min ($P = 0.02$), showing no significant changing pattern for up to 420 min. When the changes of serum neutrophil chemotactic activity were compared in five symptomatic workers with negative bronchial challenges (group II), there were no significant changes during the bronchial challenge, as shown in Fig. 2 ($P > 0.05$, respectively).

MPO level in induced sputum

Figure 3 shows the changes of MPO/albumin level in induced sputum before and 420 min after the bronchial challenges. The median value collected before the bronchial challenge were 1.9 $\mu\text{g}/\text{mg}/\text{mL}$, and post-challenge median value collected at 420 min after bronchial challenge was 10.4 $\mu\text{g}/\text{mg}/\text{mL}$ in group I patients. MPO/albumin levels

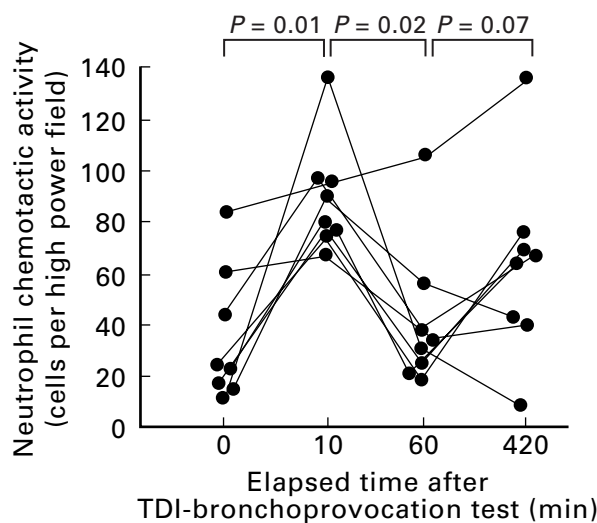


Fig. 1. Changes of serum neutrophil chemotactic activity during TDI bronchial challenge test in eight TDI-induced asthmatic subjects. Statistically significant differences were noted ($P < 0.05$, respectively).

significantly increased at 420 min after the bronchial challenges ($P = 0.02$) relative to baseline values in group I patients, while no significant changes were noted in group II subjects ($P = 0.47$).

IL-8 level in induced sputum

Table 2 shows the changes of IL-8/albumin level in induced sputum before and 420 min after bronchial challenges in

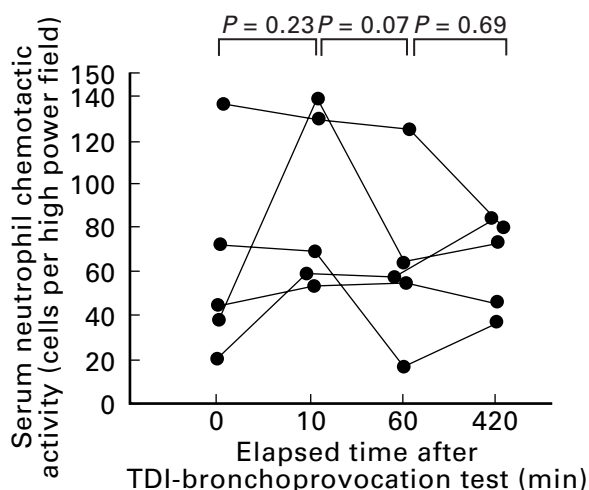


Fig. 2. Changes of serum neutrophil chemotactic activity during bronchial challenge test with TDI in five symptomatic subjects with negative bronchial challenges. No statistical significances were noted ($P > 0.05$).

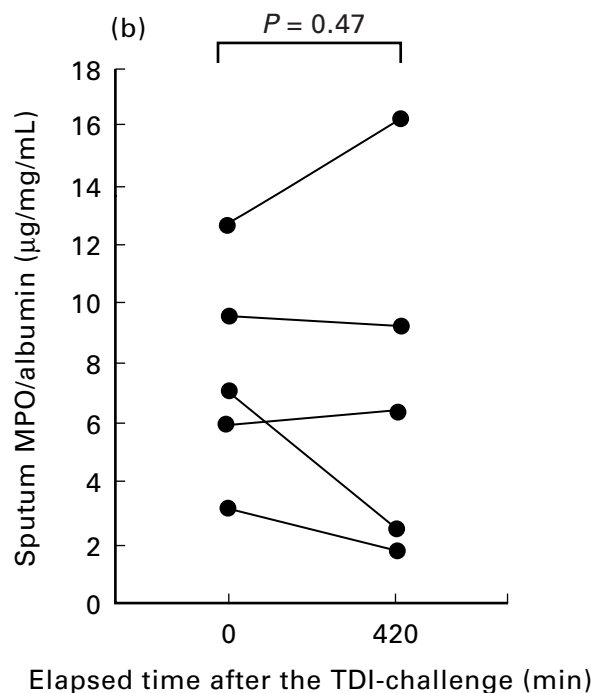
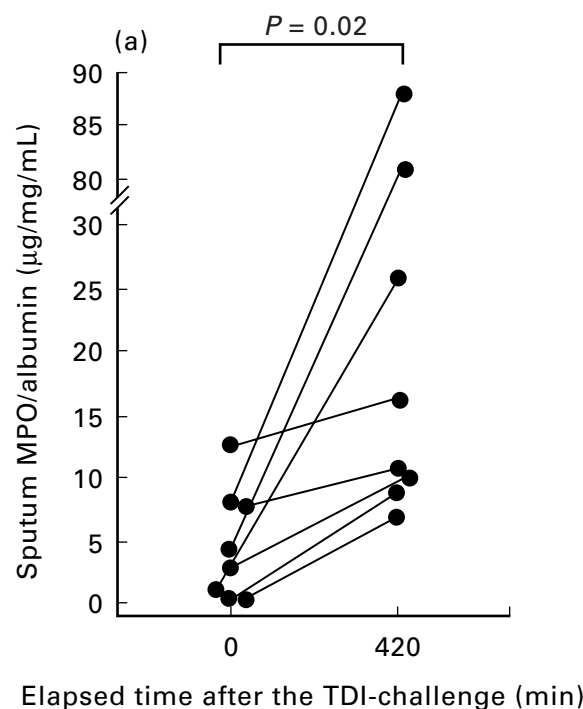


Fig. 3. Changes of myeloperoxidase (MPO) level in induced sputum before and after the bronchial challenge in eight TDI-induced asthmatic subjects showing a positive result on TDI bronchial challenge (a) and those with negative challenges (b). A significant increase of MPO/albumin relative to the baseline was noted after the bronchial challenge test ($P = 0.02$), while no significant changes in negative responders ($P = 0.47$).

Table 2. Changes of sputum IL-8 level in induced sputum before and at 420 min after the TDI bronchial challenge test in eight TDI-induced asthma subjects

Patient	IL-8/albumin (pg/mg/mL)	
	Before*	After*
1	3.40	12.3
2	1.53	8.74
3	290.3	865.1
4	51.4	120.7
5	0	6.6
6	15.4	41.9
7	19.0	33.5
8	0	6.5

* $P=0.01$, significant increase in IL-8/albumin was noted at 420 min after the TDI bronchial challenge test.

group I patients. IL-8 was abundantly present in the baseline samples of induced sputum from all group I subjects and the median value collected before the bronchial challenge test was 3.4 pg/mg/mL and post-challenge median value collected at 420 min after the challenge was 22.9 pg/mg/mL. IL-8/albumin levels increased significantly after the bronchial challenges relative to baseline values in group I patients ($P=0.01$). A significant correlation was found between percentage increase of IL-8 and post-challenge MPO level ($r=0.89$, $P=0.02$).

Discussion

TDI has been the most prevalent cause of occupational asthma in Korea [1]. However, the pathogenic mechanism is still unclear. It has been suggested that TDI may act as a hapten, in combination with protein-carrier molecules, to provoke an immune response [3–5,17]. Elevated serum-specific IgE antibodies have been detected in some subjects with TDI-induced asthma, which reflects the involvement of other immunological and/or nonimmunological mechanisms.

Recently, several studies supporting evidence of neutrophil involvement in the pathogenesis of occupational asthma have been reported. Inhalation of grain sorghum dust was found to induce increased peripheral neutrophil count and chemotactic response to grain dust [18]. *In vitro* studies revealed the release of substances presenting neutrophil chemotactic activity by bronchial epithelial cells in response to grain dust [19]. In the case of TDI-induced asthma, neutrophilia in bronchoalveolar lavage fluid had been noted in subjects with a late asthmatic reaction induced by isocyanate challenge test [11]. Our recent investigation

of bronchial mucosa applying the immunohistochemical method revealed that significantly higher numbers of neutrophils were noted in bronchial mucosa of TDI-induced asthma than in allergic asthma, regardless of whether serum-specific IgE antibodies to TDI–HSA conjugate were present or not [10]. So far, the mechanism of neutrophil recruitment in TDI-induced asthma has not been well documented. In this study, MPO levels in induced sputum and its significant increase after the bronchial challenge in positive responders, in contrast with no change in negative responders, confirmed a possible involvement of activated neutrophils in tracheobronchial secretion. Moreover, serum neutrophil chemotactic activity significantly increased 30 min after bronchial challenge relative to the baseline, and then decreased at 60 min. However, no significant changes were observed in exposed workers with negative responders on bronchial challenge testing. These findings suggest a possible involvement of neutrophils in the development of bronchoconstriction induced by TDI.

The presence of neutrophil chemotactic activity has been demonstrated in sera of individuals with asthma after provocation with allergens [20–22] or non-specific stimuli such as exercise [23]. Basically, the activity may be attributed to heat-stable and heat-labile substances. The origin of these activities is incomplete so far, although some indirect data would indicate mast cells as the origin in the case of heat-stable activity, and monocyte/macrophage for the heat-labile one [20,21]. The time course for the occurrence of serum heat-labile activity is clearly different from that of the heat-stable one. The latter activity showed a quick rise coinciding with or preceding early reduction of FEV₁, whereas heat-labile activity was demonstrated following a second peak which coincided with the late asthmatic response [24]. In this study, TDI–HSA conjugate itself did not have neutrophil chemotactic activity. The asthmatic subjects with positive challenges had a peak of neutrophil chemotactic activity at 10 min after inhalation challenge, followed by a significant decrease in activity at 60 min with no significant changes for up to 420 min. On the basis of the time course, we speculated that neutrophil chemotactic activity found in this study may be originated from mast cells, which is comparable with Sastre *et al.*'s study [11]. Further studies are needed to characterize the neutrophil chemotactic factor released after TDI inhalation.

IL-8 has been recognized as a key chemokine in inducing neutrophil recruitment and activation. Neutrophils are known not only as mere terminal effector cells, they can also affect other cells by releasing various cytokines [25,26]. Early evidence for 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 [27]. This

study showed that the IL-8 level in induced sputum significantly increased after the bronchial challenge test, compared with the baseline with concomitant increase of MPO level. Sputum IL-8 levels tended to be higher than those detected in grain dust-induced occupational asthma [28]. Moreover, there was a significant correlation between percentage increase of IL-8 and post-challenge MPO level in induced sputum. These results suggest that IL-8 released after exposure to TDI might have contributed to neutrophilic infiltration into airway mucosa. Further investigations will be needed to elucidate the clear underlying mechanism of IL-8 release upon exposure to TDI.

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 were consistent and yielded more concentrated secretions than those obtained from bronchoscopy [16,29–31]. In our study, we applied this method to detect MPO and IL-8 in tracheobronchial secretion. The albumin level in the sputum was measured to correct the dilution factor, and both MPO and IL-8 levels were presented as ratios to albumin (MPO/albumin, IL-8/albumin). The results presented in our paper suggest that sputum induction methodology might be a useful tool for investigating airway inflammation of TDI-induced asthma.

In conclusion, these findings confirm the view that activated neutrophils are involved in the development of TDI-induced bronchoconstriction, and that IL-8 may contribute to neutrophil recruitment.

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