

Elevated Interleukin-4 and Interleukin-6 in Rats Sensitized with Toluene Diisocyanate

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Abstract: In order to investigate the cytokine profile in toluene diisocyanate (TDI)-induced occupational asthma, we conducted a quantification of cytokine production in a murine model of respiratory hyperreactivity to TDI. Wistar rats were sensitized with intranasal application of 10% TDI and provoked with 5% TDI to induce airway hypersensitivity. The blood leucocytes were counted, and bronchoalveolar lavage (BAL) was performed and the cellular responses in BAL fluid were analysed. Lung histological examination was performed to investigate the inflammatory status in the airway. The production of IL-2, IL-4, IL-6 and IFN- γ in serum, BAL fluid and spleen cell were determined with ELISA kits. The cellular results demonstrated that neutrophils and eosinophils in blood were significantly increased and the total cells and each different cell, in particular eosinophils in BAL fluid were markedly increased in TDI sensitized rats. Histological analysis showed that a respiratory inflammation represented by prominent infiltration of eosinophils in central and peripheral airways was present in TDI-sensitized rats. The cytokine assays revealed that in TDI-sensitized rats, IL-4 was predominately secreted in serum, and IL-4 and IL-6 rather than IL-2 and IFN- γ were predominately secreted in BAL fluid and in spleen cell. These findings suggested that IL-4 and IL-6 are preferentially produced and may have an important role in occupational asthma induced by TDI.

Key words: TDI, Rat, Cytokine, Eosinophil, Inflammation, Occupational asthma

Introduction

Despite occupational asthma is the most prevalent occupational respiratory dysfunctions, its exact mechanisms are not well defined. Toluene diisocyanate (TDI), a low-molecular-weight compound extensively used as an industrial chemical, is known as one of the most common causes of occupational asthma. Animal models have been used to obtain greater understanding of chemical sensitizers that cause occupational asthma¹. Guinea pig models of hypersensitivity to TDI have been frequently developed in laboratories, but the immunopathologic characters of

immunized animals have not been clarified in detail. Although recently murine models of airway hypersensitivity induced by TDI have been applied to identify components of the immune response^{2,3}, the immunology, cellular response and airway pathology in the animals have infrequently been coordinately investigated. In our previous experiment on an asthmatic rat model exposed to TDI, we focused our study on the inflammatory reaction in airway and the cytokine profiles in BAL *in vivo* and in BAL-cell *in vitro*, which revealed that Th2 cytokines were preferentially produced in the airways of exposed animals⁴. However, the inflammatory process and cytokine secretion in other systems were not characterized. In this study, we established an airway hypersensitivity model in Wistar rats by intranasal

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application of TDI, and analysed the inflammatory events in blood and airway, and investigated the histological change in airway, and concurrently determined the status of secretions of an array of cytokines in blood, airway and spleen.

Materials and Methods

Animals

Six or seven-week-old male Wistar rats were used for the experiment. They were housed in the animal facility at constant temperature ($25 \pm 2^\circ\text{C}$) and humidity (50–70%) with a 12-h light period. After a week of acclimatization to our laboratory, the rats were randomly assigned to control and TDI-sensitized groups. Each group had 10 rats.

Sensitization and provocation of animals

2,4-toluene diisocyanate (TDI) (Wako Chemical Co, Japan) was used as the antigen. TDI was prepared in ethyl acetate to a concentration of 10% for sensitization and to a concentration of 5% for provocation. Under slight ether anesthesia, rats were sensitized by administration of $5 \mu\text{l}$ of 10% TDI into nostrils for seven consecutive days. After a week of rest, the rats were sensitized again for seven days. A week after the second course of sensitization, the rats were provoked by intranasal administration of $5 \mu\text{l}$ of 5% TDI. The rats in the control group were treated with vehicle ethyl acetate and provoked with 5% TDI in the same protocol. The airway hyperreactivity symptoms of rats in both sensitized and control groups were observed for 1 h after provocation.

Blood cell count and serum preparation

After the airway symptoms have been observed, rats were anesthetized by intraperitoneal injection with sodium pentobarbital, and blood was removed from cervical vein. The total leucocytes were counted after the blood was diluted at 1:10 with 1% acetic acid to lyse red blood cells. Leucocyte subtype counts were made on the blood smear followed staining with Diff-Quik (International Reagents Corp, Japan). Serum was prepared and stored at -80°C for cytokine assay.

Bronchoalveolar lavage and cell count

Bronchoalveolar Lavage (BAL) was performed with PBS at 37°C through a polyethylene tube introduced into the trachea. The BAL fluid was centrifuged at 500 g (4°C), and the supernatant was collected and stored at -80°C until cytokine determination. The cell pellet was resuspended in RPMI 1640 and counted in a hemacytometer. Cell viability was determined with 0.2% trypan blue. Differential cell

counts were made on cover glass preparations as described by Laviolette *et al.* with some modifications⁵. Briefly, a circular microscope cover glass 18 mm in diameter was placed in a flat-bottomed tube with an internal diameter of 20 mm. A $300 \mu\text{l}$ aliquot of cells at a dilution of 2×10^5 cells/ml was placed in the tube and centrifuged at 600 g for 20 min, at 4°C . After centrifugation, the fluid was gently aspirated, and the cover glass was removed and allowed to dry. The cover glass was attached to a microscope slide with nail polish, exposing the cell-bearing surface. The slide was then stained with Diff-Quik and 300 cells were examined.

Spleen cell culture

Spleen was aseptically excised, and single-cell suspension was obtained by teasing the tissue through a steel mesh into ice cold RPMI 1640. The suspension was treated with 8 g/l Tris ammonium chloride to lyse red blood cells, and then centrifuged at 200 g for 5 min at 4°C . Cell pellet was washed 3 times in RPMI 1640 and resuspended in complete RPMI 1640 (containing 20 mM HEPES, 2 mM glutamine, $100 \mu\text{g}/\text{ml}$ gentamycin, 100 U/ml streptomycin, and 10% FBS). Cell viability was determined by trypan blue, and cell suspension was enumerated with a haemocytometer. Spleen cells (5×10^5 cells/well) were cultured in a 24-well plate for 48 h at 37°C in a 5% CO_2 incubator in the presence of $5 \mu\text{g}/\text{ml}$ concanavalin A (ConA) (Sigma, St. Louis, USA). At the end of the incubation period, the supernatant fraction was collected, pooled and stored at -80°C for cytokine analysis.

Histological analysis

Lung and trachea were filled intratracheally with buffered formalin. The lung was removed, fixed and dehydrated through a series of ethanol solutions, and embedded in paraffin. $2 \mu\text{m}$ thick section was sliced and stained with hematoxylin-eosin for examination.

Cytokine assay

The selected cytokine productions of IL-2, IL-4, IL-6 and IFN- γ in serum, BAL fluid and spleen cell cultured *in vitro* were determined with commercial rat IL-2, IL-4, IL-6 and IFN- γ ELISA kit respectively, according to the manufacturer's instructions. IL-2, IL-4 and IL-6 kits were from Cosmo Bio Co., LTD. (Tokyo, Japan), and IFN- γ kit was from Endogen Inc. (MA, USA). The sensitivities of the assays were $<5 \text{ pg}/\text{ml}$, $<8 \text{ pg}/\text{ml}$, $<2 \text{ pg}/\text{ml}$ and $<2 \text{ pg}/\text{ml}$ for IL-2, IL-4, IL-6 and IFN- γ , respectively.

Statistical analysis

Mann-Whitney U test was used to analyse the cellular

Table 1. Total cells and leucocyte subtypes in blood ($\times 10^3$ cell/ml)

Group	Total cells	Leucocyte subtypes				
		Basophils	Eosinophils	Lymphocytes	Monocytes	Neutrophils
Control	15.50 \pm 1.11	0.03 \pm 0.02	0.06 \pm 0.03	13.40 \pm 0.95	0.04 \pm 0.02	1.86 \pm 0.23
TDI-sensitized	16.20 \pm 1.01	0.02 \pm 0.01	0.42 \pm 0.11*	13.59 \pm 1.11	0.04 \pm 0.01	2.10 \pm 0.19**

Each of control and TDI-sensitized groups has 10 rats. Data are means \pm SD. * and ** significantly different from control values at $p < 0.01$ and $p < 0.05$ respectively.

Table 2. Total cell and differential cell counts in BAL fluid ($\times 10^5$ cell/ml)

Group	Total cells	Eosinophils	Lymphocytes	Macrophages	Neutrophils
Control	13.25 \pm 2.90	0.04 \pm 0.01	1.23 \pm 0.40	11.74 \pm 2.56	0.09 \pm 0.02
TDI-sensitized	49.40 \pm 12.31*	1.42 \pm 0.35*	4.38 \pm 1.11*	41.37 \pm 10.60*	0.92 \pm 0.56*

Each of control and TDI-sensitized groups has 10 rats. Data are expressed as means \pm SD. *significantly different from control values at $p < 0.01$.

data in blood and BAL fluid in the control and TDI-sensitized groups, and Student's t-test was used to analyse the differences of cytokine productions between the two groups.

Results

Airway hyperreactivity symptoms

After provoked with 5% TDI, the TDI-sensitized rats exhibited irritability, sneezing and hyperrhinorrhea. The exertional breathing which was similar to asthma was observed to last 10-20 min in TDI-sensitized rats. The control rats did not show airway abnormality.

Circulating leucocyte count

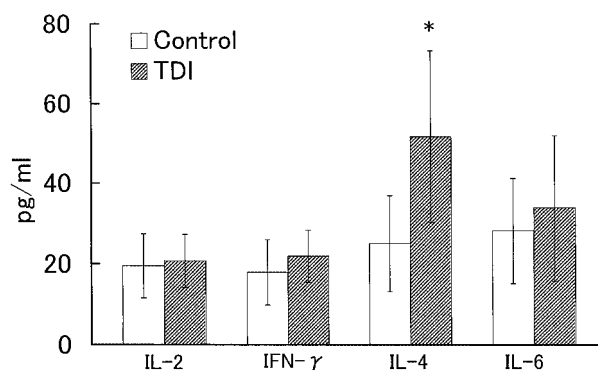
TDI exposure resulted in the increasing of the numbers of circulating neutrophil and eosinophil in blood, although the numbers of total leucocyte and basophil, lymphocyte and monocyte between the two groups were not significantly different (Table 1).

Cellular content in BAL fluid

As shown in Table 2, the total cell and each leucocyte subtype in BAL fluid in TDI-sensitized rats were significantly elevated, particularly the numbers of neutrophil and eosinophil were increased 10 and 35 times respectively compared to those in control rats.

Cytokine production in serum

As shown in Fig. 1, IL-4 concentration in serum in TDI-sensitized rats was significantly increased compared to

**Fig. 1. Cytokine concentrations in serum.**

Data are expressed as means \pm SD. *significantly different from control value at $p < 0.01$.

control rats. However, IL-2, IL-6 and IFN- γ productions in both groups were not different.

Cytokine production in BAL fluid

In BAL fluid, the concentrations of IL-4 and IL-6 in TDI-sensitized rats were significantly higher than those in control rats. Although IL-2 and IFN- γ in TDI-sensitized rats were slightly elevated compared to control rats, these differences did not reach statistical significance (Fig. 2).

Cytokine production in spleen cell

In TDI-sensitized rats, spleen cell cultured *in vitro* produced 3-fold IL-4 production and 2-fold IL-6 production compared to control rats, but IL-2 and IFN- γ productions

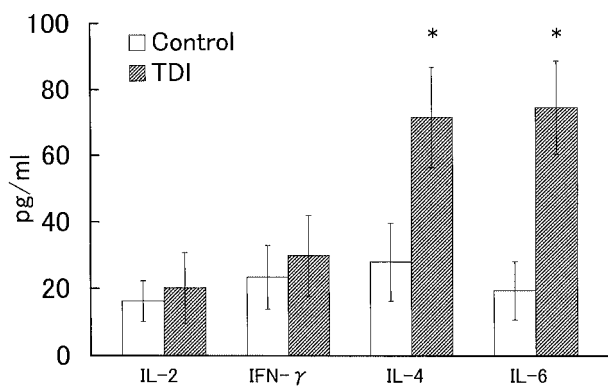


Fig. 2. Cytokine concentrations in BAL fluid.

Data are expressed as means \pm SD. *significantly different from control values at $p < 0.01$.

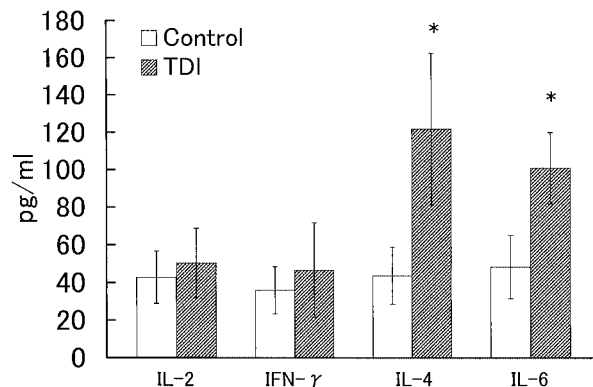


Fig. 3. Cytokine concentrations in spleen cell.

Data are expressed as means \pm SD. *significantly different from control values at $p < 0.01$.

between two groups were similar (Fig. 3).

Histological examination

As shown in Fig. 4, in TDI-sensitized rat, a prominent infiltration of central airway and peripheral airway with numerous eosinophils was observed, and goblet cells were seen in the epithelium of the central bronchi. In contrast, no major pathologic change was found in both central and peripheral airways of control rat.

Discussion

In this study, we reported a model of airway hyperreactivity in Wistar rat induced by intranasal exposure to TDI through providing the evidences of blood and airway cellular events, airway histological changes and the cytokine secretions in serum, BAL fluid and spleen cell. We observed that in TDI-sensitized rat, an inflammation represented by infiltration of eosinophils was present in airway, IL-4 was dominantly produced in serum, IL-4 and IL-6 were preferentially secreted in BAL fluid and spleen cell.

TDI is known as a potent sensitizer that causes occupational asthma in a significant proportion of subjects exposed, although the mechanisms are largely obscure. The studies have reported that bronchial asthma is a common clinically diverse condition with an appreciable inflammatory component^{6,7}. In present study, BAL cell counts revealed that an inflammation represented by markedly increased numbers of leucocytes, in particular the numbers of eosinophil and neutrophil, was present in the airways of TDI-sensitized rats. Histological examination showed that the inflammation characterized by accumulation of eosinophils was not confined in central airways, but extended to peripheral

airways. The airway inflammation was agreement with respiratory hyperreactivity symptoms which were similar to asthma. These results were consistent with those in our previous rat model exposed to TDI⁴. Eosinophil infiltration of the bronchial mucosa is known as the most striking histopathologic feature of asthma. Studies have demonstrated that the most of these infiltrated eosinophils are activated which secrete eosinophil cationic protein (ECP) and major basic protein (MBP)^{8,9}. Eosinophil products are postulated to contribute to airway hyperreactivity through damage to the airway epithelium, leaving underlying smooth muscle more susceptible to nonspecific contractile mediators, or perhaps by contributing to the airway remodeling and narrowing^{10,11}. In present rat airway hypersensitivity model to TDI, in addition to airway inflammation, sensitization with TDI resulted in an increment in the number of circulating eosinophil and neutrophil, which also indicated an inflammatory reaction occurring in the sensitized rats.

It has been reported that airway inflammation plays an important role in the pathogenesis of asthma, and that asthmatic airway inflammation is a type of cell-mediated immune response in which specialized populations of activated lymphocytes interact with other inflammatory cells through an array of cytokines¹². Th2 lymphocytes and cytokines have been considered as a key role in modulating the local inflammatory processes and in maintaining bronchial hyperresponsiveness¹³ in allergic asthmatic patients exposed to allergen such as TDI. Although animal have been used to clarify the pathogenesis of TDI-induced asthma, the cytokine profiles in animal models of asthma induced by TDI are controversial. In a mouse model exposed to TDI reported by Dearman *et al.*, Th2 type cytokines IL-4 and IL-6 were preferentially produced in lymph node cells³.

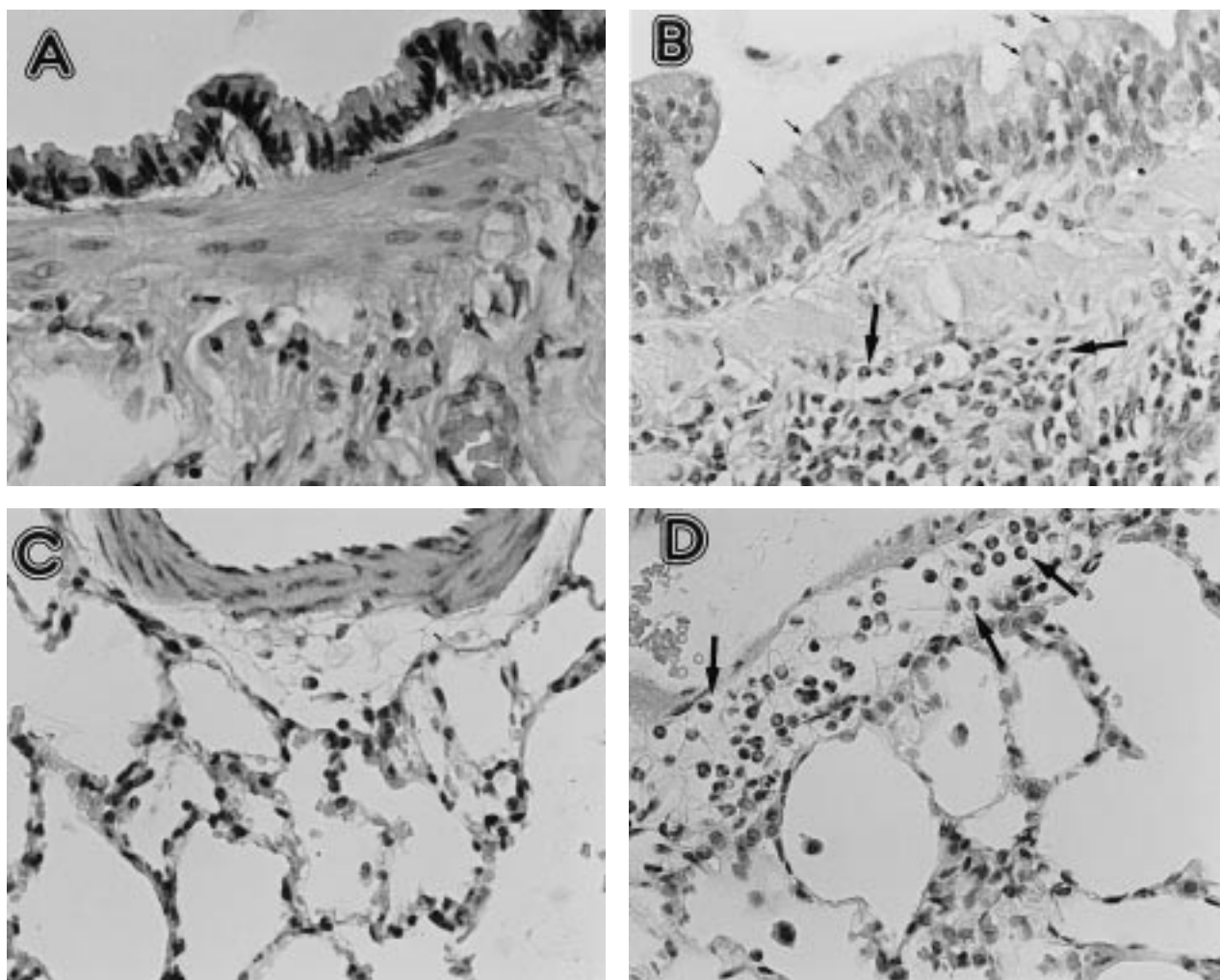


Fig. 4. Histological sections of the lungs in control and TDI-sensitized rats.

Note that both central airways (B) and peripheral airways (D) are infiltrated by eosinophils (large arrows) in a TDI-sensitized rat (B, D). A large number of goblet cells (small arrows) are seen in the epithelium of central bronchi of TDI-sensitized rat. No major pathologic change is found in control rat (A, C). Stained with hematoxylin-eosin, light microscopy; original magnification $\times 200$.

To the contrary, in a study conducted by Satoh *et al.*, the production of IL-2, IL-4, IL-5, IL-6 and IFN- γ in serum and BAL fluid between TDI-sensitized and control mice were not different¹⁴. Rat model exposed to TDI has seldom been reported, but in our previous study, we provided evidence that IL-4 and IL-6 were dominantly produced in BAL *in vivo* and BAL-cell *in vitro* after rat was exposed to TDI⁴.

In the present study, in order to further investigated the cytokine secretions not only in airway, but also in other systems, we coordinately quantified IL-2, IL-4, IL-6 and IFN- γ production in BAL, serum and spleen cell. Although IL-5 is one of the most important Th2 cytokines which promotes the accumulation and activation of eosinophils in

the allergic inflammatory sites¹⁵, we could not measure it directly because anti-rat IL-5 antibody was not commercially available. The present study showed that IL-4 and IL-6 rather than IL-2 and IFN- γ were predominately secreted in BAL fluid *in vivo* in TDI-sensitized rats. These results were agreement with our previous data that IL-4 and IL-6 were the dominant cytokines in the airways of rats exposed to TDI. Similarly to the cytokine profiles in airways, the dominant cytokines secreted in spleen cells were IL-4 and IL-6 instead of IL-2 and IFN- γ . Moreover, in blood of TDI-sensitized rats IL-4 was particularly preferentially produced. These findings, taken together, implied that Th2-type cytokines were preferentially secreted in airway system and other systems, and that these cytokines might play an

important role in airway inflammation and in immune reaction and hyperreactivity in animals with TDI sensitization. IL-4 is well known to be required for the priming of Th2 cells, it promotes the production of IgE antibodies which play a key role in the elicitation of immediate hypersensitivity reactions associated with respiratory allergy^{16, 17}. IL-6 has been reported to have activities on a wide range of cellular processes, including T-cell activation and immunoglobulin production by B cells, and thus it enhances IL-4-dependent IgE synthesis^{18, 19}.

Although the results here further supported the concept that Th2 cytokines play a principal role in the inflammation and airway hyperreactivity in asthma, the sources responsible for the increased cytokines during hyperreactivity could not be defined. The further studies are necessary to investigate the origin of the cytokines and to determine the exact role of cytokines in occupational asthma caused by TDI.

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