Cytokine Profiles in Airways of Rats Exposed to Toluene Diisocyanate

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Abstract: Cytokine Profiles in Airways of Rats Exposed to Toluene Diisocyanate: Kui-Cheng ZHENG, et al. Department of Preventive Medicine, School of Medicine, University of the Ryukyus—To investigate the status of cytokines in asthmatic airways induced by toluene diisocyanate (TDI), we established a Wistar rat model for asthma, and did a quantitative determination of cytokine production in asthmatic rats induced by TDI. Rats were sensitized with two courses of intranasal application of 10% TDI with each course for seven consecutive days, and then provoked with 5% TDI, and were followed by observation for airway symptoms. Bronchoalveolar lavage (BAL) was carried out, and total and differential leucocytes from BAL fluid were counted. Lungs were histopathologically examined for the status of inflammation in the airways. The selected cytokines, IL-2, IL-4, IL-6 and IFN-γ productions, in BAL fluid in vivo and from BAL cells cultured in vitro were quantified with ELISA kits. The results showed that the rats with TDI sensitization exhibited asthmatic symptoms. TDI exposure resulted in an increase in the total number of cells, particularly eosinophils and neutrophils in BAL fluid. Histopathological examination showed a marked infiltration of central and peripheral airways with eosinophils in TDI-exposed rats. In TDI-sensitized rats, the productions of IL-4 and IL-6 in BAL fluid in vivo were markedly increased compared to that in control rats, but production of IL-2 and IFN-γ was similar in the two groups. The cytokines from BAL cells cultured in vitro were released in a similar pattern as in vivo. These findings indicate that IL-4 and IL-6 are predominantly secreted in the airways and may play an important role in the pathogenesis of TDI-induced occupational allergic asthma.

Key words: TDI, Rat, Asthma, BAL, Airway, Cytokine, Eosinophil

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Materials and Methods

Animals

Eight-week-old male Wistar rats weighing 250–270 g obtained from Kyudou breeding laboratories (Kumamoto, Japan) were used for the experiment. The rats were kept in the animal facility at constant temperature (25 ± 2°C) and humidity (50–70%) with a 12-hr light period. Animal care was in accordance with applicable guidelines of the Ryukyu University Policy on Animal Care and Use. After a week of acclimatization to our laboratory, the rats were randomly assigned to two groups: control and TDI sensitization groups, each containing 9 rats.

Antigen

The antigen for sensitization was 2,4-toluene diisocyanate (TDI) (Wako Chemical Co, Japan). TDI was prepared in ethyl acetate to a concentration of 10% for sensitization and to a concentration of 5% for provocation.

Sensitization and provocation with TDI

Sensitization with TDI was carried out by the method reported by Tanaka, et al.4 with a slight modification. Under slight ether anesthesia, rats were sensitized by dropping 5 µl of 10% TDI into each nostril for seven consecutive days. After a week of rest, the rats were resensitized for seven days. A week after the second course of sensitization, the rats were provoked by intranasal administration of 5 µl of 5% TDI. The rats in the control group were treated with ethyl acetate and provoked with 5% TDI in the same protocol. The airway symptoms of rats in both sensitization and control groups were observed for 1 hr after provocation.

Bronchoalveolar lavage (BAL)

One hr after provocation, the rats were anesthetized with sodium pentobarbital, and a cervical incision was made and blunt dissection was performed, exposing the trachea. A 14-gauge catheter was inserted into the trachea and sutured in place. The trachea was infused with 3 ml of PBS (37°C), and the effluent was recovered by gravity drainage. The aliquot was centrifuged at 500 g (4°C), and the supernatant was collected and stored at -80°C until cytokine determination. The trachea was further infused three times with 3 ml of PBS (37°C), and the recovered fluid was centrifuged at 500 g at 4°C. The cell pellets were resuspended in RPMI 1640 and counted in a hemacytometer. Cell viability was determined with 0.2% trypan blue. Differential counts were made on cover glass preparations as described by Laviolette, et al.5 with some modifications. 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 µl aliquot of cells at a dilution of 2×10³ 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 slides were then stained with Diff-Quik (International Reagents Corp, Japan) and 300 cells were examined.

BAL-cell cultured in vitro

2 ml of BAL cells (5×10⁵ cells/ml) in complete RPMI 1640 (containing 20 mM Hapes, 2 U/ml penicillin, 100 U/ml streptomycin and 10% FBS) was dispensed into a 24-well flat-bottom Falcon plate and cultured at 37°C for 36 hr in a humidified 5% CO₂ incubator in the presence of 5 µg/ml concanavalin (ConA) (Sigma, St. Louis, USA). At the end of the incubation period, the culture supernatant was collected, pooled and stored at -80°C until cytokine assay.

Histopathological examination

Lungs were removed, without lavage, fixed in buffered formalin, dehydrated through a series of enthanol solutions, and embedded in paraffin. 2 µm thick sections were sliced and stained with hematoxylin-eosin for examination.

Cytokine assay

The production of IL-2, IL-4, IL-6 and IFN-γ in BAL fluid in vivo, and in BAL cells cultured in vitro were quantified with commercial rat IL-2, IL-4, IL-6 and IFN-γ ELISA kits, according to the manufacturer’s instructions. IL-2, IL-4 and IL-6 were from Cosmo Bio Co., LTD. (Tokyo, Japan), and IFN-γ was from Endogen Inc. (MA, USA). The detection limits of the assays were 5 pg/ml, 8 pg/ml, 2 pg/ml and 2 pg/ml for IL-2, IL-4, IL-6 and IFN-γ, respectively.

Statistical analysis

Data were expressed as means ± standard deviations (SD). Mann-Whitney U test was used to determine the differences between the control and TDI-sensitized groups in total and differential cell counts, and Student’s t-test was used to analyze the differences between the two groups in cytokine productions after the data had been logarithmically transformed. A P value of less than 0.05 was accepted as significant.

Results

Symptoms

Within 1 to 10 min after provocation, all the rats in the TDI sensitization group coughed severely and exhibited exertional breathing. The rats had their mouths open and were gasping for air. The exertional breathing was seen to last for up to 30 min in 3 rats and for up to 1 hr in 6 rats. Prolongation of the expiratory phase was observed.
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The rats also demonstrated irritability, aggression, sneezing and hyperrhinorrhea. In control rats, no airway symptoms were observed after provocation. The mean weights of the two groups before and after the experiment were not significantly different (data not shown).

**BAL cell content**

Intranasal exposure to TDI induced a pulmonary inflammatory response in the rats. The total number of cells from BAL fluid in the TDI-sensitized group was much higher than that in the control group. The microscopic glass cover technique applied to BAL cell differential counts revealed that the absolute number of each type of leukocyte in TDI-sensitized rats was significantly higher than that in control rats, particularly eosinophils were increased 45 times and neutrophils were increased 42 times in TDI-sensitized rats compared to the control rats (Table 1).

**Cytokines from BAL fluid in vivo and BAL cells cultured in vitro**

As shown in Fig. 1, IL-2 production in BAL fluid from control rats ((8.7–16.6), 3.4 pg/ml) ((min-max), SD) and TDI-sensitized rats ((9.3–26.5), 4.8 pg/ml) was not significantly different (P=0.105), and IFN-γ production in control rats ((9.9–25.6), 7.7 pg/ml) and TDI-sensitized rats ((11.5–25.3), 4.5 pg/ml) was also similar (P=0.298), but IL-4 production in TDI-sensitized rats ((19.9–70.1), 14.8 pg/ml) was significantly higher than that in control rats ((11.5–42.3), 10.1 pg/ml) (P=0.001), and IL-6 production in TDI-sensitized rats ((22.6–92.3), 24.5 pg/ml) was also significantly higher than that in control rats ((16.7–56.3), 11.6 pg/ml) (P=0.024). The concentrations of IL-2, IL-4, IL-6 and IFN-γ in the samples were higher than the detection limits for the assay kits.

As shown in Fig. 2, in BAL cells cultured in vitro, the productions of IL-2, IL-4, IL-6 and IFN-γ were generally enhanced in both TDI-sensitized and control rats after provocation.

**Table 1. Total Cell and Differential Cell Counts in BAL (×10⁵ cell/ml)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number in the group</th>
<th>Total Cell</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>11.84 ± 1.52</td>
<td>10.68 ± 1.35</td>
<td>0.96 ± 0.23</td>
<td>0.07 ± 0.03</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>TDI-sensitized</td>
<td>9</td>
<td>37.20 ± 9.10</td>
<td>30.30 ± 6.88*</td>
<td>2.73 ± 0.89*</td>
<td>2.59 ± 1.17*</td>
<td>1.32 ± 0.56*</td>
</tr>
</tbody>
</table>

Values are means ± SD, the data in brackets [ ] are percentages, * significantly different from control values at p<0.01.
the cells were cultured for 36 hr in the presence of ConA. The cytokine profiles from BAL cells cultured in vitro were in accordance with that in BAL fluid in vivo, namely, the production of IL-2 in control rats ((12.5–52.3), 11.0 pg/ml) and TDI-sensitized rats ((19.2–62.1), 12.3 g/ml), and the production of IFN-γ in control rats ((19.0–60.1), 11.1 pg/ml) and TDI-sensitized rats ((19.3–72.3), 16.1 pg/ml) was not significantly different (P=0.306 and P=0.687 respectively), whereas IL-4 production ((63.3–123.3), 22.6 g/ml) and IL-6 production ((119.0–256.3), 43.2 pg/ml) in TDI-sensitized rats were considerably greater than IL-4 production ((25.6–82.2), 19.1 pg/ml) and IL-6 production ((72.3–186.6), 31.7 pg/ml) in control rats, respectively (P=0.001 and P=0.003 respectively). The concentrations of IL-2, IL-4, IL-6 and IFN-γ in the samples were higher than the detection limits for the assay kits.

Histopathological findings
In rats with TDI sensitization, marked eosinophil accumulations were observed in the subepithelial layers of the central and small bronchi, in alveolar lumens, and in dilated blood vessels. A number of goblet cells were seen in the airways of rats with TDI sensitization, but among the control animals no major pathologic change was found (Fig. 3).

Discussion
In this study, a model of asthma in Wistar rat induced by intranasal exposure to TDI with evidence of airway symptoms, airway cellular events, airway histopathologic changes, and cytokine production in BAL fluid in vivo and from BAL cells cultured in vitro were reported. In our study, TDI-sensitized rats had asthmatic symptoms, an inflammation represented by accumulation of eosinophils in airways, and preferential secretion of IL-4 and IL-6 in BAL fluid in vivo and from BAL cells cultured in vitro.

Animal models of respiratory chemical sensitivity have been described9). The most frequently used rodent species has been the guinea pig. The Brown-Norway rat, an atopic strain of rat, has also been chosen as animal model since it favors hypersensitivity induced by chemical substances9). Several studies including our previous experiment have provided evidence of pulmonary chemical sensitivity in mice3, 5, 10, 11). In the present study we tried to establish an asthmatic model in the Wistar rat, which is used quite commonly in various experiments on toxicology. Our results revealed that the Wistar rat also provided an excellent model for the study of chemical-induced asthma, which was characterized by airway hypersensitivity and airway inflammation. Compared with the mouse model which we used in the previous study, a Wistar rat model would be more suitable for the observation of airway symptoms and for mechanistic operations such as bronchoalveolar lavage, etc.

TDI is known to induce respiratory disorders such as bronchial asthma and hypersensitive pneumonitis in workers in factories producing polyurethane plastic11).
Morphologic studies in subjects with asthma induced by TDI have demonstrated the characteristic features of allergic inflammation in the bronchial mucosa, i.e., infiltration of eosinophils and mast cells. In the present study, the cellular analysis of BAL fluid revealed that the number of eosinophils was dramatically increased in the airways, and the histopathological analysis showed that eosinophils markedly infiltrated not only the central but also the peripheral airways in asthmatic rats exposed to TDI. Our results are consistent with the data reported by other researchers who previously described the asthma models in guinea pigs induced by TDI. A number of immunohistochemical studies have revealed that most of the infiltrated eosinophils in asthma are activated.

Eosinophils are suggested to be potent proinflammatory cells because of the release of toxic granule proteins, the generation of lipid mediators such as platelet-activating factor and leukotriene C4, and participation in antibody-dependent cytotoxicity reactions, and thus are capable of degrading the pulmonary connective tissue matrix and of injuring lung cells by specific cytotoxic actions. Eosinophils are also reported to participate within cell networks as immunomodulatory cells which respond to cytokines and produce cytokines themselves. It has been indicated that there is a strong correlation between infiltration of eosinophils and airway inflammation which plays a significant role in the pathogenesis of asthma.

In our study, the local airway inflammatory processes...
were not only by the asthma symptoms but also by the accumulation of eosinophils in the airways.

Asthmatic bronchial inflammation is a specialized form of cell-mediated immunity in which lymphokines are secreted principally by activated T lymphocytes, particularly by CD4 cells. It is well known that the characteristics of immune responses are orchestrated by the activity of Th cell subpopulations and their cytokine production. Th1 cells secrete IL-2, and their soluble product, IFN-γ, which inhibits the stimulation of IgE antibody responses and the elicitation of immediate-type allergic reactions. Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 which favor the induction of acute allergic response and immediate-type hypersensitivity. IL-4 promotes the production of IgE and IL-5 promotes the development, localization, and function of eosinophils, which contribute to airway inflammation. IL-6 acts 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. Dearman et al. have reported that Th1-type cytokines (IL-4, IL-10) were preferentially secreted in lymph node cells isolated from mice exposed to TDI. Ban et al. reported that the levels of TNF-α and IL-6 in BAL were increased in guinea pigs after exposure to TDI. In our previous study we demonstrated that IL-4 and IL-5 were preferentially secreted in splenocytes and thymocytes in BALB/c mice exposed to TDI. In the present study we tried to further clarify the cytokine secretion pattern in asthmatic airways through quantitative analysis of the cytokine production in BAL fluid in vivo and from BAL cells cultured in vitro. Our results showed that IL-4 and IL-6 were the dominant cytokines in BAL fluid, and the data on BAL cells cultured in vitro confirmed this cytokine profile in vivo. Our results further indicated that Th1-type cytokines play an important role in the airway inflammatory reactions and hyperresponsiveness in chemical-induced asthma, but our results could not explain exactly why Th1-type cytokines were dominantly secreted during asthma induced by TDI in rats.

From our study we conclude that Wistar rat models would be valuable in studying TDI-induced asthma, and IL-4 and IL-6 tend to be preferentially secreted in asthmatic airways in rats, and they may play an important role in the pathogenesis of asthma. A further study is necessary to understand and to define the profiles of these cytokines during the full course of asthma and their exact roles in asthma.

References

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