Cytokine Production by Splenocytes and Thymocytes in Mice after Intranasal Exposure to Toluene Diisocyanate

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Abstract: Cytokine Production by Splenocytes and Thymocytes in Mice after Intranasal Exposure to Toluene Diisocyanate: Kui-Cheng Zheng, et al. Department of Preventive Medicine, School of Medicine, University of the Ryukyus—To determine the status and the role of cytokines in occupational asthma induced by toluene diisocyanate (TDI), we performed a quantitative analysis of cytokine profiles by means of splenocytes and thymocytes from an asthmatic murine model induced by TDI. Female BALB/c mice were treated with two courses of intranasal application of 5% TDI in ethyl acetate for 5 consecutive days each time separated by a one week rest. The control group of mice were similarly treated with vehicle. A week after the second sensitization, both groups of mice were provoked by applying 2.5% of TDI in vehicle, and nasal responses were scored for ten minutes. The results show that the TDI-sensitized group of mice exhibited the nasal allergic-like responses of sneezing and hyperrhinorrhea. Interleukin (IL)-4, IL-5 production by splenocytes, thymocytes and the total serum IgE level in TDI-sensitized mice were significantly higher than that in control mice. IL-2 production by splenocytes and IL-2, IFN-γ by thymocytes in both control and TDI-sensitized groups were not significantly different. IFN-γ produced by splenocytes was significantly higher in TDI-sensitized mice than that in control mice. In TDI-sensitized mice, the total serum IgE level was significantly positively correlated with IL-4 and IL-5 production by splenocytes and thymocytes, respectively. These findings suggest that splenocytes and thymocytes preferentially secrete Th2 type cytokines during murine asthma and these cytokines may play an important role in the pathogenesis of TDI-induced occupational allergic asthma. (J Occup Health 1998; 40: 279–284)

Key words: TDI, Allergy, Occupational asthma, Cytokine, Mice, Sensitization, Splenocytes, Thymocytes

Asthma is one of the common chronic lung diseases in industrialized countries. The prevalence of asthma has risen in recent years despite the general health improvement in the population1). Although the exact mechanisms and pathogenesis are poorly understood, there is evidence that they involve interactions among the immune system, genetic susceptibility, infections and the environment2–5). Toluene diisocyanate (TDI) is a reactive chemical widely used in the paint and plastics industries that is an important cause of occupational asthma. While the syndrome of TDI-induced asthma is well recognized, many of its mechanisms are still obscure.

Descriptions of the pathologic features of bronchial asthma are largely based on autopsy studies 6–7). Only a few studies are available on the pathology of occupational asthma induced by TDI. There is increasing evidence that the bronchial inflammation characteristic of asthma represents a type of cell-mediated immunity in which specialized populations of activated lymphocytes interact with other inflammatory cells through an array of cytokines8). Many studies on the cytokine profile of asthmatic patients have revealed that Th2 lymphocytes and cytokines play a key role in modifying the local inflammatory processes that occur after exposure to allergens, in maintaining bronchial hyperresponsiveness, and in controlling specific IgE production9). But, as far as we know, no quantified analysis of cytokine production from splenocytes or thymocytes in subjects with occupational asthma, particularly with TDI-induced asthma, has been reported.

The objectives of the present study were to determine the cytokine profile from splenocytes and thymocytes by using an asthmatic murine model induced by TDI.

Materials and Methods

Animals and Diets

Specific pathogen free four-week-old female BALB/c mice weighing 12–14 g obtained from Kyudo breeding laboratories (Kumamoto, Japan) were used for the experiment. The mice were kept in a room at constant...
temperature (25 ± 2°C) and humidity (50% to 70%) with a 12-hr light period. Animal care was in compliance with applicable guidelines from the Ryukyu University Policy on Animal Care and Use. The mice were allowed to adapt to our laboratory environment for one week before the start of the experiment, during which period they were maintained on a commercial non-purified diet (Nihon Clear, Osaka, Japan). After the period of acclimatization, the mice were randomly assigned to two groups: control and TDI sensitization group. Mice in both groups were given free access to food and water throughout the experiment period. Each group contained 9 mice.

**Antigen**

The sensitizing antigen administered was 2,4-toluene diisocyanate (TDI) (Wako Chemical Co, Japan), which is known to cause allergic asthma in laboratory animals. TDI was prepared in ethyl acetate at a concentration of 5% for sensitization and 2.5% for provocation.

**Induction of Allergy**

Sensitization was done by the method of Tanaka et al. with slight modification (Fig. 1). Mice were sensitized by dropping 2 µl of 5% TDI dissolved in ethyl acetate from an autopipette into the nostrils of the mice under slight ether anesthesia for five consecutive days. This was repeated after a week of rest. Control mice were similarly treated with the vehicle. The mice were again allowed one week of rest after which both groups were provoked with 5 µl of 2.5% TDI without anesthesia to induce nasal allergy-like symptoms. The nasal responses of sneezing, itching, watery rhinorrhea and snorting were scored for 10 min (Table 1). Hair loss was scored by observation of the extent of hair loss from the snout of mice over the period of sensitization. One week after provocation, the mice were killed by intraperitoneal administration of 10% nembutal (0.1 ml/kg body weight). Serum was removed and immediately stored frozen for later analysis.

**Cell Preparation**

Thymuses and spleens were aseptically resected, placed in sterile petri dishes containing RPMI 1640 (GIBco, BRL, Life Technologies, NY, USA), penicillin (100 U/ml) and streptomycin (100 U/ml) and stored on ice. Single-cell suspensions were obtained by teasing the tissues through 50-mesh stainless steel wire screens with the aid of disposable plastic syringe pistons into ice cold RPMI 1640. The suspensions were then washed 3 times in RPMI 1640 and resuspended in complete RPMI 1640 (containing 20 mM Hepes, 2 mM glutamine, 100 µg/ml gentamycin, 100 U/ml streptomycin, and 100 ml heat-inactivated fetal bovine serum/l) (Sigma Chemical Co., Sigma, St. Louis, USA). Cell viability was determined by trypan blue dye exclusion. Cell suspensions were enumerated with a haemocytometer and then adjusted appropriately.

**Interleukin-2, Interleukin-4, Interleukin-5, and Interferon-γ Production**

Spleen and thymus cells (5 × 10⁶ cells/well) in complete RPMI 1640 were dispensed into 96-well flat-bottom Falcon microtitre plates and cultured in triplicate for 30 hr at 37°C

<table>
<thead>
<tr>
<th>1st sensitization</th>
<th>Rest</th>
<th>2nd sensitization</th>
<th>Rest</th>
<th>Provocation</th>
<th>Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 days</td>
<td>1 week</td>
<td>5 days</td>
<td>1 week</td>
<td>1 week</td>
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</tr>
</tbody>
</table>

*Fig. 1.* Protocol for TDI treatment. Mice were sensitized with 5% TDI in ethyl acetate for 5 days, allowed a one week rest, and resensitized again for 5 days. Mice were again rested for a week after which they were provoked with 2.5% TDI. One week after provocation the mice were sacrificed. Control mice were treated with ethyl acetate but provoked with TDI.

<table>
<thead>
<tr>
<th>Nasal Response</th>
<th>Score</th>
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<tbody>
<tr>
<td>Itching</td>
<td>0</td>
</tr>
<tr>
<td>Rhinorrhea</td>
<td>0</td>
</tr>
<tr>
<td>Snort</td>
<td>0</td>
</tr>
<tr>
<td>Hair loss</td>
<td>0</td>
</tr>
</tbody>
</table>

The scores were measured for 10 min, except for hair loss which was scored over the entire period of the experiment.
in a humidified 5% CO$_2$ incubator in the presence of 5 µg/ml concanavalin A (ConA) (Sigma, St. Louis, USA). At the end of the incubation period, triplicate supernatant fractions were collected, pooled and stored at -80°C for IL-2, IL-4, IL-5 and IFN-γ analysis. IL-2, IL-4, IL-5 and IFN-γ production were quantified with commercial mouse IL-2, IL-4, IL-5 and IFN-γ ELISA kits (Endogen, Inc, MA, USA), according to the manufacturer’s instructions. The inter-assay and intra-assay CV were <10%. The sensitivities of the assays were <3 pg/ml, <5 pg/ml, <5 pg/ml, and 15 pg/ml for IL-2, IL-4, IL-5 and IFN-γ respectively.

**Total Serum IgE**

The total serum IgE levels were determined with a mouse IgE kit (Mouse IgE EIA kit Yamasa, Yamasa Shoyu Co, LTD, Japan), according to the instructions of the manufacturer.

**Statistical Analysis**

Student’s $t$-test was used to determine significant differences between means at $p<0.05$. The Pearson correlation coefficient test was used to examine the relationship between serum total IgE level and cytokine production by splenocytes and thymocytes. Probability values of $p<0.05$ were accepted as significant.

**Results**

**Symptoms**

In mice sensitized with TDI, intranasal application of 2.5% TDI induced the nasal allergy-like responses of sneezing and hyperrhinorrhea. The mice exhibited severe irritability, ruffled fur, aggression, jumping and breathing difficulty (gasping for air), but the mice in the control group were docile. During the 10 min after provocation, the mean sneezing frequency in the TDI-sensitized group of mice was 16.44 ± 1.42 which was significantly more ($p<0.05$) than that in the control group of mice (3.0 ± 0.71). The mean score for nasal responses in TDI-sensitized mice was 2.33 ± 0.5, and in the control mice was 0 (Table 2).

**Production of IL-2 and IFN-γ by Splenocytes and Thymocytes**

As shown in Table 3, the production of IFN-γ by splenocytes was significantly higher ($p<0.05$) in the TDI-sensitized group than that of the control group, but the production of IL-2 by splenocytes and IL-2 and IFN-γ by thymocytes from mice in the TDI-sensitized group and control group were not significantly different.

**Production of IL-4 and IL-5 by Splenocytes and Thymocytes**

As shown in Table 4, the production of IL-4 and IL-5 by splenocytes and thymocytes in TDI-sensitized mice were significantly higher ($p<0.05$) than in control mice.

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### Table 2. The score of nasal symptoms induced by TDI in mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nasal Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>TDI-sensitized</td>
<td>2.33 ± 0.5</td>
</tr>
</tbody>
</table>

Values are the means ± SD, n=9 for each group.

### Table 3. IL-2 and IFN-γ production (ng/ml) by splenocytes and thymocytes from control and TDI-sensitized mice

<table>
<thead>
<tr>
<th></th>
<th>Splenocytes</th>
<th>Thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Control</td>
<td>2.35 ± 0.65</td>
<td>1.92 ± 0.32</td>
</tr>
<tr>
<td>TDI-sensitized</td>
<td>2.84 ± 0.41</td>
<td>2.56 ± 0.31*</td>
</tr>
</tbody>
</table>

Values are the means ± SD, n=9 for each group. *significantly different from control values at $p<0.05$.

### Table 4. IL-4 and IL-5 production (pg/ml) by splenocytes and thymocytes from control and TDI-sensitized mice

<table>
<thead>
<tr>
<th></th>
<th>Splenocytes</th>
<th>Thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-4</td>
<td>IL-5</td>
</tr>
<tr>
<td>Control</td>
<td>39.89 ± 10.24</td>
<td>96.44 ± 13.92</td>
</tr>
<tr>
<td>TDI-sensitized</td>
<td>118.33 ± 32*</td>
<td>389 ± 96.31*</td>
</tr>
</tbody>
</table>

Values are the means ± SD, n=9 for each group. *significantly different from control values at $p<0.05$. 

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**Total Serum IgE Production**

As shown in Fig. 2, the total serum IgE concentrations in the TDI-sensitized group of mice were significantly higher (p<0.05) than those of control mice.

![Fig. 2. Serum IgE production from control and TDI-sensitized mice. Values are the means ± SD, n=9 for each group. *significantly different from control values at p<0.05.](image)

**Relationship between Total Serum IgE and Cytokines Produced by Splenocytes and Thymocytes**

As shown in Fig. 3 and Fig. 4, in TDI-sensitized mice, there was a significantly positive correlation between the total serum IgE level and IL-4 and IL-5 productions by splenocytes (r=0.86, p<0.01 and r=0.83, p<0.01 for IL-4, IL-5 respectively). Total serum IgE was also significantly positively correlated with IL-4 and IL-5 production by thymocytes (Fig. 5 and Fig. 6) (r=0.78, p<0.05 and r=0.79, p<0.05 for IL-4 and IL-5, respectively). No other correlations were found between total serum IgE and other cytokine production.

**Discussion**

Although occupational asthma is the most prevalent occupational lung disorder, many of its mechanisms are still obscure. Toluene diisocyanate (TDI), extensively used in western countries, is a potent low-molecular-weight sensitizing agent that has been associated with occupational asthma. Animal models have been...
developed to gain increased understanding of the mechanisms of chemical sensitization and asthma, but few attempts have been made to do this with low-molecular-weight sensitizers\cite{10}. In this study, we provide evidence for use of the mice model to characterize the splenocyte and thymocyte cytokine secretion after immunization to TDI. The results of our study indicated that \textit{in vivo} stimulation of a sensitized asthmatic with nasally applied TDI is associated with the activation of T lymphocytes in the spleen and thymus, and these cells preferentially produce Th2 type cytokines (IL-4, IL-5). These results are in broad agreement with the data reported previously by Dearman \textit{et al.}\cite{14} who recorded that lymph node cells (LNC) isolated after repeated topical exposure of mice to TDI display preferential secretion of Th2 type cytokines (IL-4, IL-10). To the best of our knowledge, our study is the first report of the generation and characterization of T cell cytokines from the spleen and thymus of mice with allergic asthma induced by TDI.

Airway hyperresponsiveness and chronic airway inflammation are fundamental traits of allergic asthma. The inflammatory component of this disease is characterized by increased numbers of activated Th lymphocytes, mast cells and eosinophils within the airway lumen and bronchial submucosa\cite{15-17}. Many studies have indicated a strong correlation between the number of activated CD4+ T cells and disease severity\cite{16, 18, 19}. Activated CD4+ T cells release a variety of cytokines that have been proposed to contribute to inflammation and airway hyperresponsiveness. The types of cytokines released by activated CD4+ T cells from allergic asthmatic individuals appear to fit one of the two basic patterns of cytokine expression described in human and murine CD4+ Th cell clones. Th1 cells are characterized by increased secretion of IL-2 and IFN-\gamma, whereas the Th2 subset of CD4+ T cells preferentially secrete IL-4, IL-5, IL-6, IL-9 and IL-10\cite{20}. Examination of bronchoalveolar lavage (BAL) fluid cells and supernatants from allergic asthmatics has shown the existence of a Th2-like cytokine pattern\cite{21}. The relative proportions of cells expressing Th2 cytokines in BAL fluid or biopsies are increased in symptomatic more than in asymptomatic asthmatic individuals\cite{22, 23}, suggesting that Th2 cytokine release contributes to airflow obstruction. Th2 cytokines may contribute to allergic reactions and therefore may play an important role in the inflammatory reactions and airway hyperresponsiveness in allergic asthma. The studies have revealed that BAL and bronchial mucosal T cells from allergic asthmatic patients produce higher levels of IL-4 and IL-5 than do cells from normal controls\cite{21-23}. Our results are also consistent with these previous studies. IL-4 in particular has been shown to be a primary determinant of Th2 cell differentiation\cite{24} and is also essential for the production of IgE\cite{25} which can mediate antigen-induced mast cell degranulation. High serum levels of IgE are characteristic of allergic asthmatic individuals\cite{25}, indicating a likely role for mast cell mediator release as a significant component of allergen-induced airway constriction. Mast cells release a variety of preformed and newly synthesized mediators, which are potent bronchoconstrictors, in response to antigen stimulation\cite{26}. IL-4 may also contribute to eosinophilia by promoting the increase in endothelial vascular cell adhesion molecule-1, which controls the attachment and migration of eosinophils. IL-5 promotes the differentiation, recruitment, activation and survival of eosinophils\cite{27}, which are consistently increased in blood and airways of allergic and nonallergic asthmatic patients compared with normal subjects. Eosinophils may contribute to airway hyperresponsiveness by releasing mediators that induce epithelial injury and bronchoconstriction, including major basic protein, leukotrienes and platelet-activating factor\cite{28, 29}. IgE production is exquisitely sensitive and controlled by Th2 cells. Th1 cells secrete IL-2 and IFN-\gamma and turn off IgE synthesis, whereas Th2 cells turn on IgE synthesis.

This study shows that in mice, TDI preferentially increases the secretion of Th2 type cytokines, and this animal model may shed further light on the mechanisms involved in TDI-induced occupational asthma. Since Th2 cytokine and IgE production may play an important role in the pathogenesis of occupational allergic asthma, an effective therapy for allergic airway reactions may be to prevent the development of the Th2 cytokine pattern and inhibit the release of IgE. Although animal data should not necessarily be extrapolated to TDI-induced occupational asthmatic individuals, information obtained from such studies may be potentially transferable or serve as a basis for the study of immunocorrecting properties of TDI-induced occupational asthma.

References

6) Dunnill MS. The pathology of asthma, with special