Airway Inflammation and Bronchial Remodelling in Toluene Diisocyanate-exposed BALB/c Mouse Model

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Abstract
Toluene diisocyanate (TDI), a highly reactive industrial chemical, is one of the leading causes of occupation-related asthma in industrialized countries. The pathogenesis of TDI-induced asthma, however, remains not fully understood, in part due to lack of appropriate animal models. Twenty five female BALB/c mice (age: 8 weeks) were randomly divided into 5 groups: Ovabumin (OVA); OVA peptide amino acid residues No. 323–339 (Pep); TDI; alum and physiological saline. Mice were intraperitoneally injected with 25 µg OVA or pep absorbed on 300 µg alum, 300 µg alum or saline on days 0, 7 and 14. For the TDI group, mice were sensitized subcutaneously with 20 µl neat TDI on day 0; 20 µl of TDI in olive oil (1:10) on days 7 and 14; on days 21–23. Then each group was challenged intranasally with 20 µl of 1% OVA, 1% Pep, 1% TDI, 10% alum and saline respectively. On day 28, mice were killed under pentothal anesthesia. The results demonstrated that neutrophil-dominant inflammation with a few eosinophil infiltration occurred in the peri-bronchial and peri-vascular regions of the lungs. This was accompanied by hyperplasia/hypertrophy of cells lining the airways and mucus production as shown by HE staining. Positive immunohistochemical MBP staining in parenchyma was also shown. Th2 cytokine IL-4 and IgE production were significant increased 5 days after last challenge while IFN-γ level was below the detection limit. Conclusion: the clear elevation of IL-4 and IgE could allow to conclude a possible Th2-like dominated allergic response in TDI-exposed BALB/c mouse model.

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closely associated to TDI exposure and in conjugated form can be a useful marker for progress of TDI-asthma [12–14]. Maestrelli et al. reported that increased levels of Th2 cytokines were detected in the airways and bronchial mucosa of TDI asthmatics [15]. However, some authors observed Th1-like response characterized by an increase in the number of neutrophils and the levels of IFN-γ and IL-8 [16–18].

Because the cellular mechanisms responsible for diisocyanate-induced asthma and the determinants of exposure are not fully defined, diagnosis and control have been difficult. To help addressing these issues, we developed a murine model of toluene diisocyanate hypersensitivity, in which we used intraperitoneal injection for sensitization and intranasal administration to challenge the airways.

Materials and methods

Experimental animals. Female BALB/c mice (approximately 20 g and 8 weeks old) were obtained from Vivarium animal house, Haukeland University Hospital (Bergen, Norway). They were kept in a conventional animal house, Haukeland University Hospital (L.-Z. Sun et al.). They received tap water and pelleted pathogen-free food (Special Diet Services, Witham, Essex, England) ad libitum. All experiment procedures were approved by the local Ethical Committee for Animal Experiment.

Chemicals. Toluene diisocyanate (2,4 to 2,6-isomers; ratio, 80:20; lot S36694 311) was obtained from Merck-Schuchardt, Hohenbrunn, Germany. Ovalbumin (electrophoretic purity 99%, lot 81F-8230) was purchased from Sigma Chemical Company, St Louis, MO, USA. An Ovalbumin peptide amino acid residues No. 323–339 (Pep) was synthesized by semi-automatic peptide synthesizer (LKB Biochrom, Ltd, Cambridge, England) as described before by Johnsen and Elsayed [19]. Imject® alum (lot E164 107) was obtained from Pierce Biochnology Inc., Rockford, IL, USA.

Sensitization protocol. Twenty-five mice were randomly divided into five groups: (a) Ovalbumin (OVA); (b) OVA 323–339 (Pep) [19]; (c) toluene diisocyanate (TDI); (d) alum control; and (e) saline control.

Animals were allowed to acclimatize for 1 week and then were actively sensitized by intraperitoneal (i.p.) injections of 25 µg of OVA absorbed on 300 µg of alum, 25 µg of Pep absorbed on 300 µg of alum and on days 0, 7 and 14. For the TDI-mice the sensitization was done by subcutaneous injection with 20 µl of neat TDI on day 0, 20 µl of TDI in olive oil (1:10) on days 7 and 14. From days 21 to 23, the mice were challenged intranasally with 20 µl of 1% OVA in saline, 1% Pep in saline and 1% TDI dissolved in ethyl acetate:olive oil (1:4) respectively. Alum and saline control groups were sensitized and then challenged in a similar manner as OVA group. Five days after last challenge, the mice were killed by right ventricular exsanguination under pentothal anaesthesia (50 mg/kg body weight, i.p.).

Extraction of bronchoalveolar lavage fluid (BALF). Shortly after exsanguination, the trachea was cannulated and the lungs of the animals were then lavaged 3 times with aliquots of 0.5 ml sterile saline per mouse. The lavage fluid collected was centrifuged at 4000g to remove cells and then stored at −20 °C until it was analysed for cytokines and IgE levels.

Quantification of IgE. Total IgE in sera and the BAL fluid were quantified with a sandwich ELISA protocol provided by mouse IgE ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA). Brieﬂy, the plates were coated with afﬁnity-puriﬁed goat anti-mouse IgE antibodies (incubation for 1 h at 37 °C), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies. The plates were developed by addition of tetramethylbenzidine (TMB) and read in the ELISA plate reader (Labsystem Multiskan Bichromatic, Helsinki, Finland) at a wavelength (λ) of 450 nm. The kit detection limit was 250–3.9 ng/ml.

Quantification of IL-4 and IFN-γ. IL-4 and IFN-γ in sera and the BAL fluid were quantified by a sandwich ELISA protocol (R&D Systems, Minneapolis, MN, USA). Buffers and diluents used between steps in this assay were provided by the same company. Brieﬂy, 96-well plates were coated overnight with the capture antibody at 4 °C and then blocked for 1 h with 1% BSA in phosphate buffer saline (PBS) for 30 min at room temperature (RT). The threefold diluted sera or undiluted BAL fluid samples and appropriate dilutions of a standard IgE preparation were added into the wells, and the plates were incubated for 1 h at RT. Sample blank wells received buffer instead of sera or BAL fluid and were treated identically. The bound IgE was detected with polyclonal goat anti-IgE antibodies (incubation for 1 h at 37 °C), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies. The plates were developed by addition of tetramethylbenzidine (TMB) and read in the ELISA plate reader (Labsystem Multiskan Bichromatic, Helsinki, Finland) at a wavelength (λ) of 450 nm. The kit detection limit was 250–3.9 ng/ml.

Histology. After the lavage, the lungs and heart were removed en bloc and inflated with a similar volume of 10% buffered formalin (600 µl) at a constant pressure and then immediately soaked in the same solution for approximately 24 h, and thereafter embedded in paraffin [20].
Lung tissue sections (4 μm) were stained with haematoxylin and eosin (HE). Immunohistochemistry was performed with rabbit polyclonal antibody against murine major basic protein. Briefly, 4-μm lung tissue sections were deparaffinized, followed by quenching of endogenous peroxidase activity, blocking with 1% normal goat serum and sequentially incubated with rabbit anti-major basic protein (MBP) (1:1000) and then incubated with biotinylated swine anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark) diluted 1:500 in tris-buffered saline, respectively, for 60 min at 25°C. Sections were incubated first with avidin–biotin–peroxidase complex (DAKO) and then with diaminobenzidine for the development of a coloured reaction product before being counterstained with haematoxylin [21, 22]. Pictures were acquired with LEICA DMLB light microscope (Leica Microsystems GmbH, Wetzlar, Germany) and LEICA DC 300 camera, thereafter imported into Adobe Photoshop 7 (Adobe Systems Incorporated, San Jose, CA, USA).

Morphometric analysis. Bronchial measurements were made at 250× magnification using LEICA Q500 MC Qwin system (Leica Microsystems GmbH). Bronchia with a diameter less than 250 μm that presented a smooth circular and oval profile were selected. As schematically illustrated in Fig. 1, the measurements were made across the shortest and longest axis of the elliptical profiles at the location where the cell borders appeared sharp to minimize the influence of tangential sectioning. Measurements include the lumen diameter (B, b), the diameter of the airway between the outermost layers of smooth muscle (A, a), and the thickness of the bronchial wall from the base of the columnar epithelium to the outer limit of the adventitia (C, c). On a subset of airways (not sharing an extensive length of their adventitia with arteries), axes of the elliptical profiles were measured. Five mice from each group were enrolled in this measurement. C+c was expressed as the thickness of the airway wall for group differences analysis. The differences between individual groups were tested by an ANOVA statistical analysis for multiple comparison.

Data analysis. Values are presented as mean ± SD. ANOVA statistical analyses were performed using alum and saline as reference category, and post hoc tests with adjustments for multiple testing by the approach of Dunnet’s T3 where equal variances within groups are not assumed. Overall values P < 0.05 were considered to be significant. All tests were two-tailed. Data were analysed with SPSS statistical software (SPSS Inc., Chicago, IL, USA).

Results

None of the animals exhibited noticeable distress or significant tachypnea during the period of study, there was no mortality.

Cytokine and total IgE levels in serum and BALF

Five days after last intranasal challenge, blood was collected from control and exposed mice. The serum was analysed for total IgE and cytokines, IL-4 and IFN-γ levels (Table 1). There was a statistically significant increase in IL-4 level in the mice intraperitoneally sensitized and thereafter intranasally challenged by TDI, OVA and Pep, respectively, compared with saline or alum controls.

<table>
<thead>
<tr>
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<th>TDI</th>
<th>OVA</th>
<th>OVA 323–339</th>
<th>Reference</th>
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<tbody>
<tr>
<td>IL-4 (pg/ml)</td>
<td>73.3 ± 41.9*</td>
<td>37.3 ± 21.4*</td>
<td>72.9 ± 42.0*</td>
<td>Alum 0 Saline 0</td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>73.3 ± 41.9*</td>
<td>37.3 ± 21.4*</td>
<td>72.9 ± 42.0*</td>
<td>7.3 ± 4.7</td>
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<tr>
<td>IgE (ng/ml)</td>
<td>98.1 ± 16.0*</td>
<td>91.3 ± 9.2*</td>
<td>7.9 ± 7.1</td>
<td>8.7 ± 7.3</td>
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<tr>
<td>IFN-γ (pg/ml)</td>
<td>UD</td>
<td>UD</td>
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*ANOVA, compared with alum and saline control, P < 0.01. OVA, ovalbumin; UD, under detection limit.
When compared with alum and saline controls, 13- and 11-fold increase of serum total IgE were, respectively, found in TDI-treated mice while 12.5- and 10.5-fold were found in OVA-treated animals. As for Pep group, no increase of IgE was found when compared with the control animals. Statistically no significant difference was found between two control groups.

Non-detectable levels of IL-4 and IFN-\(\gamma\) in BALF were obtained under the experimental conditions used.

Histology

Histology of the lung tissue was performed 5 days after final challenge; the saline- and alum-exposed mice demonstrated normal lung morphology. In contrast, histological sections of lung tissue from TDI-exposed mice exhibited pulmonary inflammation, infiltrating neutrophils accompanied by macrophages, lymphocytes and a few eosinophils were mainly observed in the peri-bronchial and peri-vascular regions of the lungs. The increased neutrophils should be responsible for the cell recruit-ment in lung. Comparatively the mice sensitized by OVA demonstrated a typical eosinophil-dominant inflammation as well as Pep-treated mice (eosinophils were visualized by Congo red staining). Thickening of the bronchial wall and increased mucus production were shown by HE staining in TDI-, OVA- and Pep-exposed mice (Fig. 2). For the 4-\(\mu m\) bronchial lung sections incubated with anti-MBP, there were large number of neutrophils in the parenchyma, and intense MBP staining of the apical surfaces of respiratory epithelial cells for the animals treated by OVA, Pep and TDI. In contrast, fewer neutrophils and eosinophils infiltration were observed in saline and alum control animals, and even less MBP staining around the airways (Fig. 3).

Airway wall alternations

On light microscopic examination, the walls of the bronchia from the sensitized mice were thickened when
compared with control mice. TDI-treated mice showed, at least partly, a similar change in airway remodelling as Pep-treated mice and OVA-treated animals (Fig. 4).

Discussion

Asthma is a chronic respiratory disease characterized by the presence of reversible airway constriction and AHR to various stimuli [23]. AHR is a cardinal feature of the disease in which acute airway narrowing is easily elicited by non-specific stimuli, such as exercise, cold air or inhalation airway irritants, such as histamine or metacholine [24]. Underlying the clinical manifestations is a acute-on-chronic inflammation of airway mucosa, with degranulation of mast cells, recruitment of eosinophils and neutrophils, as well as accumulation of activated T lymphocytes and other chronic inflammatory cells [25]. Several studies of airway inflammation in non-occupational asthmatic subjects have reported heterogeneity of cell counts. Wenzel et al. reported two pathophysiological subtypes amongst severe asthmatics, i.e. the ones with and without evidence of eosinophil infiltration in bronchial biopsy specimens [26, 27]. More recent studies
where airway inflammation has been assessed non-invasively using induced sputum on diverse range of patients have shown predominant neutrophilic airway inflammation in some patients with severe asthma. Whether these changes reflect the severity of the disease or the effect of treatment was unclear [28, 29].

Although animal models are not always closely reflective of human responses, it could certainly improve our understandings of the cellular and molecular mechanism associated with TDI-induced allergic disorders [30]. TDI is capable of inducing different types of immune reactions, depending on T-cell polarization toward the helper type 1 (Th1) or helper type 2 (Th2) cells. Th1 cells promote cell-mediated immunity and are defined by their secretion of cytokines, mainly IFN-γ. Th2 cells are recognized by the secretion of interleukins, such as IL-4, IL-5 and IL-13, which support humoral immune response. In experimental studies, the divergence might be because of different animal experimenta-
tion conditions, e.g. sensitization way and dose applied [31]. Up to now, concerning the mechanism for TDI asthma, especially the Th1 or Th2 type responses, the published results remain controversial [31]. BALB/c mouse is frequently employed in studies of respiratory allergy. In case of occupational asthma, this model has commonly been used for studies for Th2 phenotype-induced diseases.

Previous morphological examination of airway tissue from patients who died of severe bronchial asthma has revealed abnormalities in the airways, which was thought to be, in part, irreversible [32]. These include goblet cell hyperplasia, sub-epithelial fibrosis and smooth muscle cell hyperplasia/hypertrophy. It is generally accepted that tissue remodelling is a process of wound healing for the maintenance of homeostasis after various injuries. These processes normally contribute to the repair of injured tissues, not only morphologically, but also functionally. However, the results of chronic airway inflammation may induce airway remodelling which may differ from wound healing, leading to airway narrowing and flow limitation. Th2 cells produce IL-4 which is closely related to the promotion of IgE production. IL-4 is also well known as a key cytokine in the development of T cells, especially Th2 type [33].

The main objective of this study was to elucidate the events involved in airway remodelling on the basis of a newly established animal model at our laboratory.

We demonstrated that repetitive allergen and TDI airway exposure induced marked increases in the inflammatory leukocytes, especially eosinophils and neutrophils in lung tissue and IL-4 secretion. In contrast, the IFN-γ level was low and undetectable under the experimental method used. Paralleling IL-4 production and IgE synthesis significantly increased after exposure both in TDI and OVA mice. In case of OVA 323–339 sensitized mice, low IgE titer was found. OVA 323–339, known to be MHC Ia-binding peptide, did not enhance IgE synthesis compared with the intact molecule. Possible explanation for this lower IgE in OVA 323–339 may be contributed to variable genetic subsets of BALB/c mice MHC, affinity of the ligand or carriers needed for a successful in vivo immune reaction [19].

Respiratory responses to TDI as well as MDI [34] only occur upon a previous contact with the same antigens. As generally known, the frequency and the concentration of sensitization prior to challenge affects the subsequent response to intranasal exposure to TDI in a complicated pathway [35]. Moreover, strain differences are known in the patterns of mRNA expression of cytokines or receptors, for example, BALB/c mice tend to exhibit higher IL-4 mRNA expression than C57BL/6 mice even when treated with Th1-predominant allergens, such as dinitrochlorobenzene (DNCB) and trimellitic anhydride (TMA) [36, 37]. Conversely, C57BL/6 mice show higher IFN-γ levels with the application of DNCB than does the BALB/c strain, namely C57BL/6 mice and BALB/c mice are Th1 and Th2 predominant respectively [38, 39].

These results reported are in line with previous reports: BALB/c mice are more likely to present Th2 dominant response, for which the underlying mechanisms are still obscure; however, partly attributable to the BALB/c mouse genetic control of IL-4 production [36]. In this study, we have focused on the specific immune response of chemically induced hypersensitivity. Cell composition data from BAL and airway function measurements, for new experiments, will be reported separately. Additionally better timing of autopsies and a revised approach for a semi-quantitative grading for the histological sections on the basis of arbitrary scores, are to be considered. Nevertheless, few additional controls would be necessary for further validation of the model, e.g. non-sensitized and sensitized but not challenged groups.

Although the present model may not demonstrate complete features of TDI-induced occupational asthma, the findings demonstrate that the development of airway remodelling, including hyperplasia/hypertrophy of the cell lining in the airways and the hyper-secretion of the mucus, probably is the result of Th2-like IgE-dependent neutrophil-dominant inflammation. In TDI-exposed allergic BALB/c mouse model, the observed neutrophil-dominant reaction is different from Th2-dominated IgE-dependent eosinophil inflammation which is commonly developed in OVA-induced murine asthma models. TDI-induced inflammation is composed of infiltrating neutrophils, macrophages and lymphocytes, which is consistent with the reports of TDI asthma in humans. Unlike humans, however, eosinophils could be very minimal or even absent [40, 41].
Similarly, in workplaces, TDI-induced asthma may result from a previous contact with TDI, some of which might initiate allergic sensitization associated with type 2 cytokine production and high IgE levels [31]. Taken together, this study indicates that BALB/c mouse as a Th2-like model of TDI-induced airway allergy may, in several aspects, mimic occupational TDI asthma in humans.

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References


