

Importance of inflammatory and immune components in a mouse model of airway reactivity to toluene diisocyanate (TDI)

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Summary

Background Nearly 9 million individuals are exposed to agents in the workplace associated with asthma, and isocyanates represent the most common cause of occupationally induced asthma.

Objectives Nonetheless, the immunological mechanisms responsible for isocyanate-induced asthma are not clear. A murine model for toluene diisocyanate (TDI) asthma is described and employed to examine inflammatory and immune components that may be involved in the disease.

Methods Groups ($n = 6$) of C57BL/6J and athymic mice were sensitized by subcutaneous injection (20 μ l on day 1, 5 μ l on days 4 and 11), and 7 days later challenged by inhalation (100 p.p.b., days 20, 22 and 24) with TDI. Twenty-four hours following the last challenge the tracheae and lungs were examined for histological changes as well as for the expression of Th1, Th2 and pro-inflammatory cytokines. Mice were also examined for airway reactivity to methacholine challenge and for specific and total IgE and IgG antibodies.

Results TDI sensitization resulted in increased reactivity to methacholine challenge as well as a significant inflammatory response in the trachea and nares of wild-type mice, but not in the athymic mice nor in the lungs of the C57BL/6J mice. Airway inflammation was characterized by inflammatory cell influx, goblet cell metaplasia and epithelial damage. Histological changes in the trachea were accompanied by increased mRNA expression of interleukin (IL)-4, tumour necrosis factor α , lymphotoxin β , lymphotactin and Rantes, as well as TDI-specific IgG antibodies and elevated levels of total IgE. IgE-specific antibodies were not detected with this exposure regimen but were produced when the TDI concentrations were increased.

Conclusions These studies provide a unique murine model for occupational asthma that generates both inflammatory and immune mediators similar to those occurring in TDI-induced asthma in humans.

Keywords: airway hypersensitivity, occupational asthma, toluene diisocyanate

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Introduction

Asthma is a chronic respiratory disease characterized by the presence of reversible airway constriction and airway hyper-responsiveness (AHR) to various stimuli. Isocyanates, such

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as toluene diisocyanate (TDI), are the most common cause of occupational asthma, with over 100 000 workers in the USA exposed every year and 5–10% of these developing asthma. Isocyanate-induced asthma has been difficult to diagnose and control, in part because the biological mechanisms responsible for the disease and the determinants of exposure have not been well defined and validated animal models do not yet exist. In humans it is characterized by specific airway hyper-responsiveness to chemical challenge as well as an increase in non-specific hyper-responsiveness [1,2]. TDI-induced asthma shares many characteristics of atopic asthma, manifested by a latency period, persistent airway hyper-reactivity and severe airway inflammation involving the presence of activated T cells, eosinophils, neutrophils and mast cells [1,3,6]. Even months following removal from exposure, airway epithelial changes and inflammation persist [7]. It has been suggested that multiple mechanisms may be involved in the pathogenesis of isocyanate-induced asthma, including immunological, inflammatory, pharmacological and neurogenic factors [1,5,7–9].

Attempts to establish an immunological etiology have received considerable attention. Patients with allergic asthma resulting from exposure to high molecular weight chemicals are typically atopic, with at least two-thirds producing IgE antibodies to the aeroallergens [7,10,11]. Furthermore, asthma prevalence is greater among subjects with high serum IgE levels. In contrast, it is estimated that only 5–30% of isocyanate-asthmatic patients produce specific IgE antibodies and their presence may have little to do with disease status or severity [10,12,14]. Lack of IgE-mediated immunity, however, does not eliminate an immunological mechanism and increasing evidence supports involvement of cell-mediated processes involving CD4⁺ and CD8⁺ lymphocytes in occupational asthma [5,8,9].

Guinea pig models, historically used to study TDI-induced asthma, display both immediate and late-onset airway responses and dose-dependent IgG antibody-specific responses [15–18]. In the present studies we describe a T lymphocyte-dependent murine model for TDI asthma which displays significant airway inflammation and hyper-reactivity as well as TDI-specific antibodies. This model manifests similarities to both previously described guinea-pig models [11,18] as well as humans with isocyanate-derived asthma [14,19,20] and should provide a valuable model to study mechanisms of isocyanate-induced asthma.

Materials and methods

TDI exposure and tissue collection

Female wild-type, athymic (nu⁺/nu⁺) C57BL/6J mice, approximately 6–8 weeks old, were obtained from Jackson

Laboratory (Bar Harbor, ME, USA). Upon arrival the mice were quarantined for 2 weeks and acclimated to a 12-h light–dark cycle. Animals were housed in microisolator cages in pathogen-free and environmentally controlled conditions at NIOSH facilities in compliance with AALAC-approved guidelines. Food and water were provided *ad libitum*.

TDI (80 : 20 molar mixture of 2,4 : 2,6: isomers) was provided by Bayer, USA (Pittsburgh, PA, USA). Sensitization was performed by sub-cutaneous injection with 20 µL of neat TDI on day 1 followed by 5 µL injections on days 4 and 11 (5 µL TDI brought up to 20 µL in olive oil). Control mice received an equivalent volume of vehicle. Challenge via inhalation (1 h, 100 ppb) was carried out on days 20, 22 and 24 following methods previously described [16,21]. Briefly, TDI concentrations in the chamber were generated by passing dried air through an impinger that contained 4 mL TDI [22]. The TDI concentration in the chamber was adjusted by varying the amount of air entering the impinger. This generation system produces TDI vapor free of TDI aerosol [23]. Real-time chamber atmosphere monitoring was performed using an AutostepTM continuous toxic gas analyser (Bacharach, Inc., Pittsburgh, PA, USA) with its probe placed ≈ 5 inches above the animals. The sensitization and challenge regimen resulting in specific IgE antibodies in separate studies had sensitization occurring on days 1, 3 and 5 (vs. days 1, 4 and 11) and inhalation challenge at a dose of 150 ppb (as opposed to 100 ppb). Due to overt toxicity to the mice, this more rigorous regimen was not pursued.

Twenty-four hours after the last challenge, the mice were killed and bronchoalveolar lavage fluid (BALF), blood, lungs, mediastinal lymph nodes, nares and tracheae were collected. Additional groups of mice were utilized for BALF collection in order to not adversely affect tissues isolated for histological assessment and RNA isolation. Tissues were immersed in 10% neutral buffered formalin, nares were decalcified and the tissues embedded in paraffin and stained with haematoxylin and eosin for blinded histopathological assessment. To obtain bronchoalveolar lavage (BALF), mice were anaesthetized, exsanguinated and intubated with a 20G cannula positioned at the bifurcation. Each mouse was lavaged three times with 1.0 mL of sterile HBSS. A 0.1-mL sample of BALF was used for cytospin preparations. The slides were fixed and stained with Diff-Quick (VWR, Pittsburgh, PA, USA) and differential cell counts were obtained using light microscopic evaluation of 300 cells/slide. Total cell counts were performed with a haemocytometer. In other experiments, the trachea and lungs were collected, frozen in liquid nitrogen and stored at –80 °C for reverse-transcriptase-polymerase chain (RT-PCR) and ribonuclease protection analyses.

Antibody detection

Total serum IgE was measured using a sandwich ELISA as previously described [24]. Briefly, plates were coated with 5 µg/mL of rat monoclonal anti-mouse IgE (PharMingen, San Diego, CA, USA). Serial two-fold dilutions of test sera (starting at 1 : 5) were added and incubated with peroxidase-conjugated goat anti-mouse IgE (1 : 1000, Nordic Immunological Laboratories, Capistrano Beach, CA, USA) and developed with ABTS (2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid]) substrate. Serum IgE concentrations were derived from a standard curve obtained using murine monoclonal anti-DNP IgE (Sigma, St Louis, MO, USA; starting dilution 1 : 500). Hapten-specific antibodies were detected by coating plates with 50 µg/mL of TDI-mouse serum albumin conjugate in 0.1 M carbonate buffer, pH 9.6 [24]. Test sera were added (serial two-fold dilutions starting at 1 : 5) and the plates were incubated with peroxidase-goat anti-mouse IgG (1 : 400, Sigma), goat anti-mouse IgE (1 : 1000, Nordic Immunological Laboratories), or goat anti-mouse IgG1 and IgG2a (1 : 400, The Binding Site, Birmingham, UK) and developed with ABTS substrate.

Airway hyper-reactivity

Airway responsiveness was assessed using a single chamber whole body plethysmograph (Buxco, Troy, NY, USA). A spontaneously breathing mouse was placed into the main chamber of the plethysmograph, and pressure differences between the main chamber and a reference chamber were recorded. The airway reactivity was expressed as enhanced pause (Penh), a calculated value that correlates with measurement of airway resistance, impedance and intrapleural pressure; $Penh = (Te - Tr / Tr) \times Pef / Pif$, where Te = expiration time, Tr = relaxation time, Pef = peak expiratory flow and Pif = peak inspiratory flow [25]. In the plethysmograph, mice were exposed for 5 min to nebulized PBS to establish baseline values and subsequently to increasing concentrations of nebulized methacholine (0–50 mg/mL, 1.0 mL in PBS) using an AeroSonic ultrasonic nebulizer (DeVilbiss, Somerset, PA, USA). After each concentration, recordings were taken for 5 min. The Penh values measured during each 5-min sequence were averaged and expressed as increase over baseline Penh values following PBS exposure.

Semi-quantitative reverse-transcriptase-polymerase chain (RT-PCR) reaction

Tissues were collected and total cellular RNA was extracted using the Qiagen RNeasy kit® (Qiagen, Valencia,

CA, USA) according to the manufacturer's instructions. cDNA was synthesized as described previously [26]. PCR primers for murine G3PDH, INFγ, IL-4 and IL-5 were purchased from Clontech (Palo Alto, CA, USA), and TNFα primers were purchased from Stratagene (La Jolla, CA, USA). Samples of cDNA were amplified by PCR using a GeneAmp PCR System 9600 DNA Thermal Cycler™ (Perkin Elmer, Foster City, CA, USA). For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 35 and 38 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength as previously described [27]. If necessary, the concentrations of cDNA were readjusted (normalized to G3PDH) and the PCR repeated. When appropriate, the specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA that generated restriction fragments of the expected size (data not shown). Amplified PCR products were separated electrophoretically on 1.5% agarose gel (UltraPure, Sigma), scanned directly with an Eagle Eye II digital imaging system (Stratagene, La Jolla, CA, USA) and quantified using ImageQuant software (Storm, Molecular Dynamics, Sunnyvale, CA, USA).

Ribonuclease protection assay (RPA)

RNA samples were quantified by RPA using the RiboQuant® kit (PharMingen). The dsDNA multiprobe templates mCK-3b and mCK-5 were purchased from PharMingen. ³²P-labelled cRNA probes were produced from dsDNA templates using the MAXIscript T7 kit from Ambion (Austin, TX, USA) according to the manufacturer's instructions. Samples were electrophoresed on an acrylamide sequencing gel, and protected fragments were quantified using a phosphor imaging system and ImageQuant software (Storm, Molecular Dynamics).

Statistical analysis

All studies were replicated with representative data shown. For statistical analysis, standard one-way analysis of variance (ANOVA) was used for multiple group comparisons. Student's two-tailed unpaired *t*-test was used to determine the level of difference between two experimental groups and *P* < 0.05 was considered a statistically significant difference.

Results

Mice were sensitized to TDI as described in the methods section and 24 h later AHR to methacholine challenge was assessed. A significant increase in AHR occurred in TDI-sensitized mice at the 25 and 50 mg/mL concentrations of methacholine when compared with sensitized athymic mice

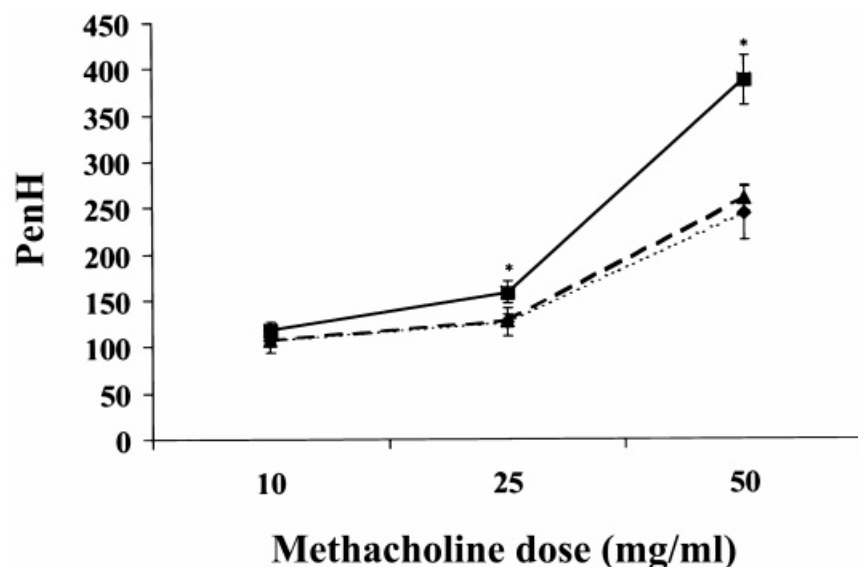


Fig. 1. AHR in TDI-sensitized mice. Twenty-four hours after the last TDI sensitization, naive (◆), TDI-exposed wild-type (■) and TDI exposed athymic mice (▲) were assessed for methacholine reactivity. The change in PenH values from baseline was determined in response to increasing doses of inhaled methacholine. *Significantly different from control and athymic groups ($P < 0.05$, $n = 6$, $\pm$ SEM).

or mice treated with vehicle alone (Fig. 1). There were no significant differences between the responses of vehicle-treated and sensitized mice to challenge with 10 mg/mL of methacholine.

Airway inflammation is a characteristic feature of the

Table 1 Summary of pathological changes induced by TDI exposure in C57BL/6J and athymic mice^{ab}

Tissue alterations	Naive C75BL	Exposed C75BL	Athymic
<i>Nares</i>			
Exudate	0	3	0–1
Goblet metaplasia	0	4	1
Inflammation	0	2	0
<i>Trachea</i>			
Cilia loss	0	2	0
Goblet metaplasia	0	1	0
Cytoplasmic extensions	0	2	0
Inflammation	0	1	0
Cell piling	0	2	0
<i>Lymph node</i>			
Activation state	0	3	1
Germinal cell formation	0	3	1

^aHistopathological changes were assessed 24 hours after the last TDI inhalation challenge.

^bValues are expressed on a 0–5 scale, with 0 representing no changes, 1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe and 5 = severe. Mean individual severity within a group was calculated by adding severity scores of all animals and then dividing that by the total number of animals.

asthmatic response to TDI and is considered a key manifestation of underlying bronchial hyper-responsiveness. While TDI-sensitized and challenged mice did not reveal appreciable histological changes in the lungs, both the tracheae and nares from sensitized mice exhibited a significant inflammatory response manifested by infiltrating neutrophils, lymphocytes and macrophages (Table 1). In addition, degenerative cellular changes were noted in the tracheae and nares, including loss of cilia, goblet cell metaplasia, septal exudate, cytoplasmic extensions and cell piling. To investigate the role of T lymphocytes in

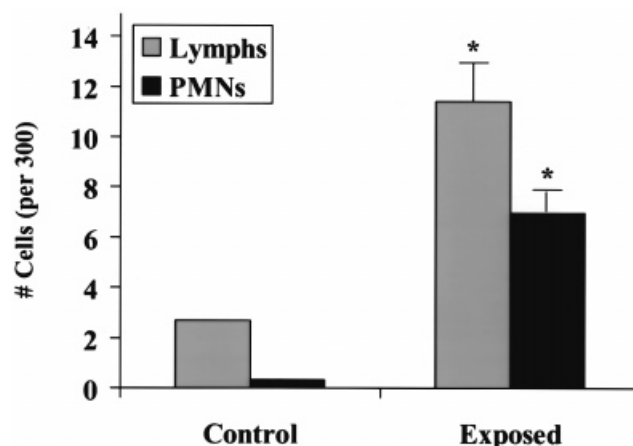


Fig. 2. Cellular constituents of BALF in TDI-challenged mice. BALF was collected 24 h after the last TDI inhalation challenge. Cytospin preparations were examined for cellular content. Differential cell counts were obtained using light microscopic evaluation of 300 cells per slide. *Significantly different from control group ($P < 0.05$, $n = 6$, $\pm$ SEM).

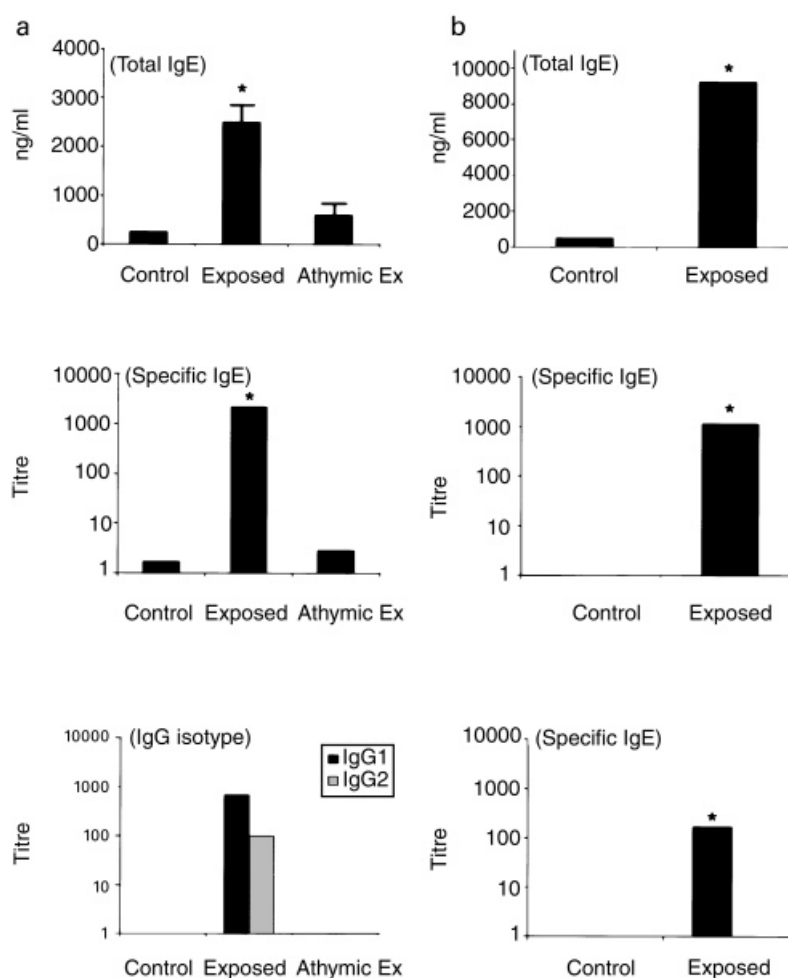


Fig. 3. Serum antibody titres to TDI. Serum was collected from control and sensitized mice 24 h after the last TDI inhalation challenge. (a) Total IgE antibodies, specific IgG antibodies, and specific IgG1 and IgG2a antibodies following the normal dosing regimen. (b) Total IgE antibodies, specific IgG antibodies and specific IgE antibodies following the higher dosing regimen. *Significantly different from control group ($P < 0.05$, $n = 6$, $\pm$ SEM).

TDI-induced asthma, athymic mice were included in the experimental design. As summarized in Table 1, athymic mice demonstrated little, if any, histopathological sequelae indicative of inflammation or degenerative changes in the nares or trachea when compared with wild-type exposed animals.

Cellular constituents in the BALF were affected by sensitization and challenge despite the lack of histological changes in the lungs of TDI-exposed wild-type mice (Fig. 2). While the predominant cell type in the BALF remained the macrophage, TDI-exposure induced 20-fold and 4-fold increases in neutrophils and lymphocytes, respectively, in the C57BL/6J mice. There was no evidence of eosinophil infiltration in control or experimental animals.

Twenty-four hours after the last TDI challenge, blood was collected and the serum analysed for total IgE levels as well as for TDI-specific IgE and IgG antibodies (Fig. 3a). TDI-exposed animals demonstrated elevated levels of total IgE and TDI-specific IgG antibodies. A mixed IgG

response was observed, with IgG1 antibodies being the more predominant isotype produced when compared with IgG2a (Fig. 3a). IgE-specific antibodies were not detected with this dosing regimen but were detectable when the inhalation exposure concentrations were increased and timing of dose changed (Fig. 3b). Inflammatory changes, primarily neutrophils and macrophages, were detected both in the bronchi and alveoli at these higher TDI exposures (data not shown). Antigen-specific IgG antibodies were not detected in the sera of athymic mice, although total IgE serum concentrations were slightly elevated following TDI treatment, albeit not significantly from naive athymic levels.

RNA was isolated from the tracheae and lungs of mice 24 h after the last challenge and the relative expression of IL-4, TNF α and INF γ mRNA was determined by RT-PCR to assess the changes in cytokine expression following TDI exposure (Fig. 4). IL-4 expression was highly induced and INF γ reduced in the tracheae of sensitized mice when compared with vehicle-treated controls. Cytokine transcript

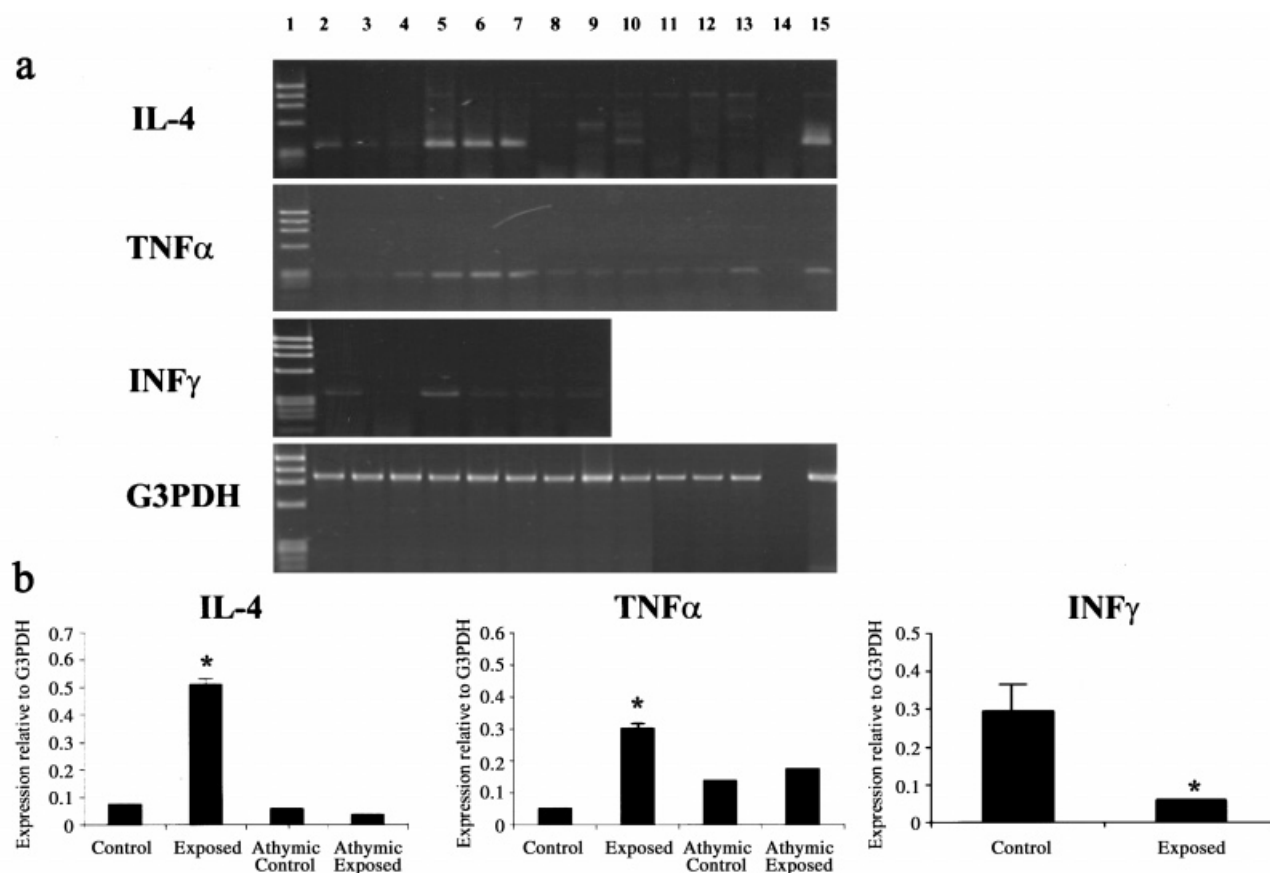


Fig. 4. TDI induces increased IL-4 and TNF α expression while suppressing INF γ expression in tracheae of C57BL/6J but not athymic mice. Twenty-four hours following the last inhalation challenge, RNA was isolated from tracheae and RT-PCR was performed using IL-4, TNF α , INF γ and G3PDH specific primers. (a) Ethidium bromide stained 1.5% agarose gels representative of three experiments and groups of $n = 12$. Lane 1, molecular weight markers; lanes 2–4, control mice; lanes 5–7, TDI-exposed mice; lanes 8–10, athymic control mice; lanes 11–13, athymic TDI-exposed animals; lanes 14–15, commercial negative and positive controls. (b) Gels were scanned with a digital analysis system and the PCR products were quantified. Data are expressed relative to G3PDH expression ($n = 12$, $\pm$ SEM).

levels in athymic-sensitized mice were similar to those of control mice. A relative increase in TNF α mRNA transcripts was also observed in the tracheae of sensitized wild-type mice, that was reduced to almost control levels in athymic mice. Low-level constitutive expression of IL-4 and INF γ occurred in the lung and was not altered by TDI exposure (data not shown). However, with the higher dosing regimen the cytokine pattern was similar in the lungs to that in the tracheae, with increases in IL-4 expression and decreases in INF γ expression, though not to the extent as observed with the tracheae (data not shown). IL-5 transcripts were not detected in either the tracheae or lungs of the exposed animals. To extend these observations on additional inflammatory cytokines and chemokines, RPA was performed on pooled mRNA from tracheae and lungs (Fig. 5). A five-fold, two-fold and two-fold increase in TNF β , lymphotactin (LT) β and TNF α , respectively,

occurred in the tracheae of TDI-sensitized mice. Consistent with the inflammatory cell influx in the upper airways and cellular increase in BALF, a four-fold and two-fold increase was noted in the expression of the chemokines Rantes and LT β , respectively. No significant changes were observed in the tracheae among the different treatment groups for the other inflammatory cytokines and chemokines. There were no treatment-related changes in the expression of any cytokines or chemokines examined in the lungs (data not shown).

Discussion

The diagnosis of isocyanate-induced asthma is largely based upon variable airflow obstruction and hyper-reactivity to non-specific (e.g. methacholine) challenge. Both epidemiological observations and experimental studies suggest that

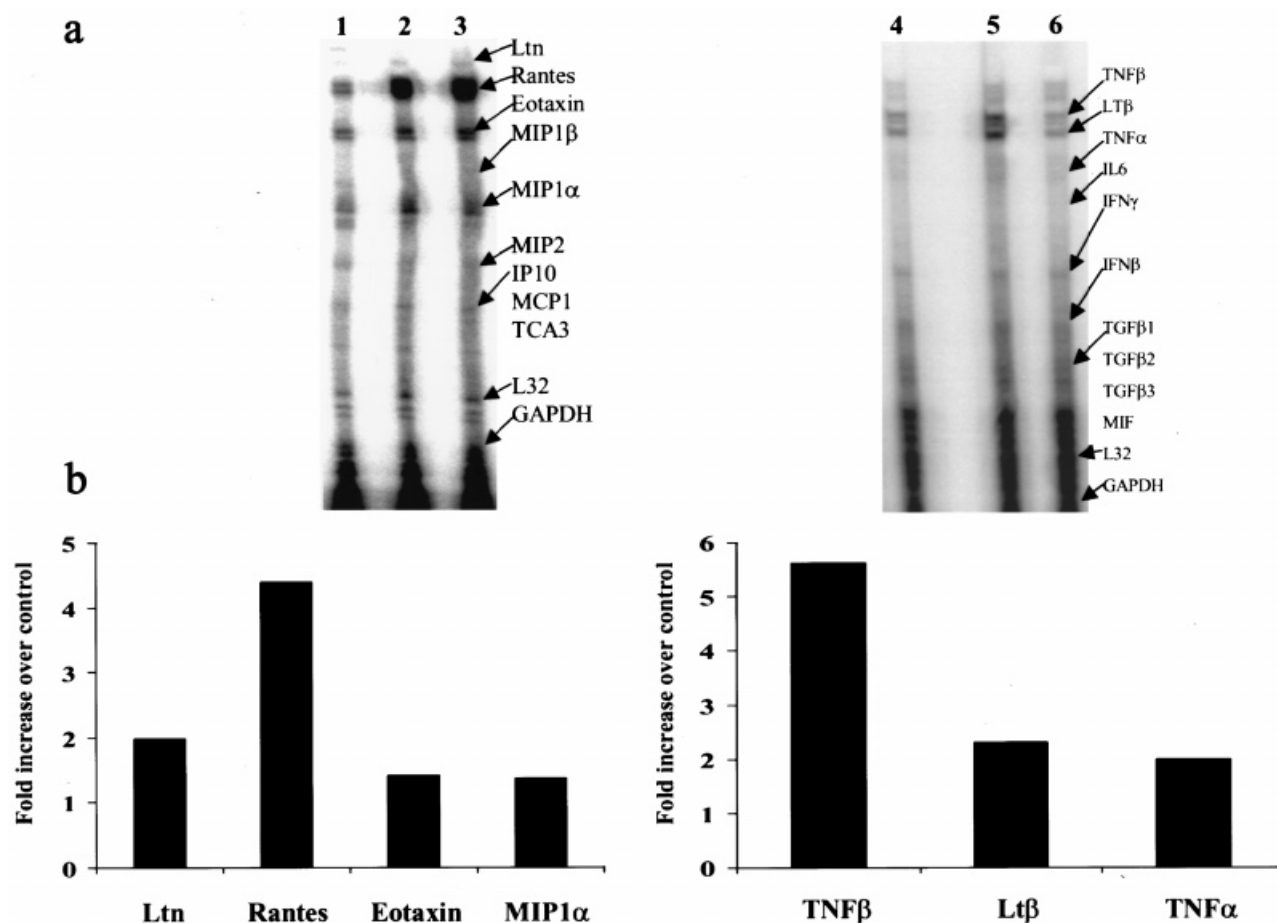


Fig. 5. Inflammatory cytokine expression in tracheae as determined by ribonuclease protection assay (RPA). Twenty-four hours following the last TDI inhalation challenge, total tracheae RNA was isolated, pooled and RPA was performed. (a) The protected fragments were separated on a polyacrylamide gel, which was dried and exposed to a phosphorimaging screen. Lane 1, control mouse; lanes 2–3, TDI-exposed mice; lane 4, control mouse; lane 5, TDI-exposed mouse; lane 6, TDI-exposed athymic mouse. Lanes 1–3 were run with the mCK-5 probe and lanes 4–6 with the mCK-3b probe. (b) Bands were quantified using a phosphorimager and data are expressed relative to L32 expression and as fold increase over control values.

classical IgE-mediated mechanisms are not operative in isocyanate-induced asthma. In this respect, recent estimates indicate that only 5–30% of isocyanate-asthmatic patients produce specific IgE antibodies [18,19,28] and such antibodies bear no relationship to disease occurrence or severity. This is in contrast to asthma caused by most high molecular weight compounds such as latex and ragweed proteins, which occurs typically in atopic patients, of which at least two-thirds demonstrate allergen-specific IgE antibodies.

Although lung pathology and eosinophilia were minimal, the current mouse model of TDI asthma demonstrates key hallmarks of the clinical disease, such as inflammation and AHR. It differs from previously described mouse models [9,24,29–36] in that responses are elicited following inhalation challenge. Consistent with the clinical picture,

we also observed elevated IgE levels and TDI-specific IgG antibodies in sensitized, challenged animals. TDI-specific IgE antibodies, observed only at relatively high exposure levels, were not a prerequisite for AHR. An increase in total IgE levels occurs in isocyanate-sensitized workers [37], and specific IgG antibodies have at least been shown to reflect exposure [12,13,38]. In addition, a direct association between TDI-specific IgG antibodies and the concentration of inhaled TDI has been shown in guinea-pigs [16], although IgG antibodies do not reflect lung hypersensitivity [16,39].

Although IgE-mediated immunity may not be a major factor in TDI-induced asthma, it appears likely that T cell immunity plays a significant role. This is evidenced in the current studies by the absence of AHR and pathology in athymic mice following TDI exposure. Lymphocyte

transfer and kinetic studies have also indirectly supported a role for cell-mediated immunity as lymphocytes, but not IgE, were shown to be a prerequisite for TDI asthma [9]. It is well established that T lymphocytes release cytokines which modulate eosinophil recruitment and adherence causing airway inflammation, and patients with TDI-induced asthma have increased numbers of circulating CD8⁺ [40] and CD25⁺ T cells, which express IL-2R [41]. This suggests that TDI exposure not only causes airway inflammation but also the recruitment of activated leucocytes and inflammatory cells through T cell mechanisms. Consistent with a role for T cell mechanisms, it has been shown that while CD4⁺ T cell clones predominate in the BALF of patients with atopic (extrinsic) asthma, the majority of lymphocytes isolated from individuals with TDI asthma represent CD8⁺ T cells [42].

Cytokines play an integral role in the initiation, propagation and persistence of inflammatory and immunological processes in asthma. Atopic asthma is often associated with Th2-biased responses and can be adoptively transferred with Th2 cells [43,44]. IL-4, a Th2 cytokine, promotes mucus secretion and goblet cell hyperplasia [45–48]. Th1 cytokines, including IL-2 and IFN γ , appear less important in allergic asthma but can induce mediators responsible for airway inflammation. Following TDI sensitization, we observed elevated IL-4 expression in the trachea that was accompanied by decreased expression of IFN γ . This would suggest that the TDI response is preferential to a Th2-type cytokine profile despite using C57BL mice, a predominate Th1 mouse strain. This is in contrast to airway response changes observed with Balb/c mice, intranasally challenged to TDI, where the short-term exposures are believed to be reflective of a delayed-type-hypersensitivity reaction, which is characterized by Th1 responses [36]. IL-5 transcripts were not detected in either the tracheae or lungs of the exposed animals, consistent with the absence of eosinophils within the involved tissues. One hallmark of TDI-asthma is goblet cell metaplasia within the affected airways. In this respect, IL-4 has been shown to directly induce the differentiation of epithelial cells into goblet cells that are responsible for mucin secretion [49]. The expression of inflammatory cytokines, represented by TNF α , Rantes and LT β , was also enhanced in tracheae following TDI exposure. Inflammatory cytokines such as TNF α can be assumed to play multiple roles in occupational asthma. For example, TNF α can potentiate mucosal inflammation in the airway epithelium and stimulate mucin secretion in airway epithelial cells via the generation of nitric oxide [50]. TNF α also enhances Th2 cell transendothelial migration, suggesting that inflammatory cytokines are important in Th2 responses [51]. In this respect a crucial event in the inflammatory

response is the recruitment and influx of leucocytes into the affected tissues.

In summary, a mouse inhalation model is described which shows striking similarities to TDI-induced occupational asthma in humans. Predominant effects include AHR, histopathological changes in the upper airways, and elevated total IgE and TDI-specific IgG antibodies. Epithelial changes, represented by goblet cell metaplasia, loss of cilia, cytoplasmic extensions and cell piling, were accompanied by mild inflammation composed of infiltrating neutrophils, macrophages and lymphocytes, and are consistent with reports of TDI asthma in humans [7]. Unlike humans, however, there was an absence of eosinophils. The lack of pathological changes and specific IgG antibodies in athymic mice strongly suggests an immunological component to TDI-asthma. This model should prove invaluable to further investigation of the pathophysiological mechanism of the disease.

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