Animal models for diisocyanate asthma: answers for lingering questions

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Purpose of review

Diisocyanates are the leading cause of occupational asthma, the most commonly reported lung disease associated with the workplace. Clinical studies have implicated the immune system in the pathogenesis of occupational asthma, but ethical and moral issues prevent mechanistic investigations in humans. For this reason, the development and characterization of animal models are germane to further understanding of diisocyanate occupational asthma and to identify avenues for therapeutic intervention. This review will highlight important features of existing experimental animal models with emphasis on new developments.

Recent findings

Experimental animal models of diisocyanate occupational asthma have demonstrated an immunological basis for the disease. Mice can be sensitized by dermal or respiratory exposure, suggesting that either exposure route may be important in the workplace. Recent findings show that sensitized mice develop airway hyperreactivity and inflammation, reflective of human disease. The transfer of lymphocytes or serum from sensitized mice can cause clinical disease in naive mice. Transgenic animals have identified a role for specific immunity, including the involvement of T-helper type 1/2 responses as well as CD4 and CD8 T cells in diisocyanate occupational asthma. Recent animal models have shown that sensitization can occur through subchronic inhalation of vapor-phase diisocyanate at levels as low as 20 ppb.

Summary

Recent progress using animal models has been instrumental in furthering current understanding of the involvement of the immune system in disease pathogenesis. The demonstration of diisocyanate occupational asthma in a murine model after subchronic inhalation exposure at relevant exposure levels should provide opportunities for more accurate risk assessment data.

Keywords

animal model, diisocyanate, diphenyl-methane diisocyanate, hexamethylene diisocyanate, murine model, occupational asthma, toluene diisocyanate

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Abbreviations

AHR	airway hyperreactivity
HDI	hexamethylene diisocyanate
MCP-1	monocyte chemoattractant protein type 1
OA	occupational asthma
TDI	toluene diisocyanate
Th	T-helper
VEGF	vascular endothelial growth factor

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Introduction

Occupational asthma (OA) is the most frequently reported occupational respiratory disease in industrialized nations [1]. Diisocyanates, highly reactive low molecular weight chemicals, are the leading cause of OA, a disease that accounts for nearly 10% of all adult-onset asthma [2]. The three major diisocyanates encountered in the workplace include toluene diisocyanate (TDI), diphenyl-methane diisocyanate and hexamethylene di-isocyanate (HDI), which are used in a variety of industries including polyurethane foam manufacturing, auto body painting and repair and plastics manufacturing. It is estimated that as many as 5% of workers exposed to diisocyanates develop asthma, which may persist indefinitely even in the absence of continued exposure. Clinically, diisocyanate OA displays similar manifestations to those present in allergic asthma, suggesting a common immunopathogenesis, although clinical data have highlighted several important differences, such as a low prevalence of specific IgE antibodies, mixed T-helper (Th) type 1/2 responses and the involvement of CD8 T lymphocytes in diisocyanate OA. The development of animal models has been instrumental in furthering the current understanding of the immune mechanisms involved in allergic asthma [3]. New target molecules and mediators as well as new therapeutic approaches for allergic asthma have been identified using animal models. The purpose of the present review is to highlight the major features of diisocyanate OA in humans and to summarize recently developed animal models.

Human diisocyanate asthma: clinical outcomes and pathophysiology

Diisocyanate OA is often characterized by a variable latent period, consisting of months to years of exposure before the development of symptoms in workers [4••]. Once sensitized, low-level exposures, even those below permissible workplace limits, can induce the clinical

onset of disease [5•]. Patients with diisocyanate OA can develop immediate, late or dual asthmatic reactions after chemical exposure. In addition, patients often develop persistent airway hyperreactivity (AHR) to non-specific stimuli that can last for years even in the absence of continued exposure, and the complete recovery of lung function may never be achieved [6,7,8••]. Recent evidence demonstrates a correlation between the recovery of AHR and the persistence of eosinophilic inflammation in diisocyanate OA with persistent inflammation associated with poor recovery of lung function [9]. Histopathological features of the disease include goblet cell metaplasia, mucus hypersecretion, upper and lower respiratory tract inflammation, consisting of leukocytic infiltration of the airway mucosa and leukocyte extravasation, leading to lumenal eosinophilia and neutrophilia [10•]. There is also evidence demonstrating airway remodeling in diisocyanate asthmatic individuals, characterized by subepithelial thickening (collagen deposition) and fibrosis [11]. Airway remodeling may be an important determinant of the persistence of OA. It is evident that the clinical manifestation of OA exhibits many similarities to that of allergic asthma.

Although immune mechanisms are presumed, the pathophysiological mechanisms responsible for diisocyanate-induced OA are not as well defined as those of allergic asthma. In allergic asthma, the recruitment of CD4 T lymphocytes to the lung and the production of Th2-type cytokines including IL-4, IL-5 and IL-13 occur. These and other Th2 cytokines are responsible for eosinophil recruitment, mucus hypersecretion and IgE production [12]. In diisocyanate asthmatic patients, activated T lymphocytes and eosinophils infiltrate the airways [10•] and IL-4 and IL-5 can be detected in airway mucosa [13,14]. However, clinical studies have suggested that OA may involve both Th1/Th2 cells, as cytokines from both cell types are found after the *in vitro* stimulation of patients' lymphocytes [15–17]. Furthermore, the presence of activated CD8 T lymphocytes in peripheral blood and airways are found in diisocyanate OA [18,19]. These T lymphocytes represent a $CD3^+/CD4^-/CD8^+$ population expressing the γ/δ T-cell receptor [20,21••]. Other evidence that the etiopathogenesis of diisocyanate OA may differ somewhat from that of allergic asthma is the low prevalence (5–30%) of specific IgE antibodies [22,23], and the association with the chemokine, monocyte chemoattractant protein type 1 (MCP-1). Regarding the latter, Lummus *et al.* [16] showed that MCP-1 is induced upon the restimulation of peripheral leukocytes from diisocyanate asthmatic patients, and its production is more predictive than specific antibodies for the identification of diisocyanate OA [24••].

Genetic polymorphism studies demonstrated that the glutathione-*S*-transferase gene family is associated with the prevalence of diisocyanate OA [25•,26]. More recently, a genetic variation in *N*-acetyltransferase, another antioxidant enzyme system, was shown to be associated with the risk of diisocyanate OA, with slow acetylators being more susceptible [27•]. The identification of nitric oxide synthase in the airway mucosa of diisocyanate OA patients suggests an additional role for nitric oxide, another reactive radical associated with inflammation [28]. Such studies suggest that the genetically determined response to oxidative stress is a disease-specific risk factor for disease development.

Animal models of diisocyanate occupational asthma

Animal models of disease are invaluable to investigate mechanisms and identify therapeutic targets. Murine models of allergic asthma have led to the identification and characterization of leading treatment modalities in humans, including leukotriene antagonists [29] and more recently bacterial products [30–32]. Animal models of OA are in their infancy compared with models of allergic asthma, but significant advances have been made. The following discussion will highlight recent animal models of diisocyanate OA with emphasis on experimental advances using such models.

The first animal studies of diisocyanate OA were conducted in guinea pigs, and identified this class of chemicals as respiratory toxins capable of acute airway irritation as well as immune-mediated sensitization [33]. Guinea pig models display many of the clinical features of human diisocyanate asthma, including AHR, epithelial injury, neutrophilic inflammation [34–36], airway eosinophilia [37,38], and a late phase asthmatic reaction [38]. Studies using guinea pigs have also shown that exposure to skin or airways, common routes of exposure in the workplace, can lead to respiratory and dermal sensitization [39].

Early studies using murine models demonstrated the involvement of the immune system in diisocyanate OA, as topical exposure to TDI induced the production of TDI-specific IgE antibodies [40–42] and contact hypersensitivity [43]. It was later shown that CD4 and CD8 T lymphocytes were important effector cells in these responses [44]. However, these early models lacked evidence of respiratory inflammation. Newer murine models of diisocyanate OA developed before August 2002 have been reviewed by Redlich *et al.* [45]. Recently, Scheerens *et al.* [46•] demonstrated that epicutaneous sensitization to TDI led to increased tracheal hyperreactivity to carbachol. Male BALB/c mice were exposed to 1% TDI epicutaneously on days 0 and 1, followed by intranasal challenge 8 days later with 20 μl

1% TDI. Importantly, lymphocyte involvement in the tracheal hyperreactivity was demonstrated by adoptive transfer experiments. Changing the sensitization schedule to 6 weeks, with skin exposure to TDI on days 0, 7, 14, 21, 28 and 35, followed by intranasal challenge, resulted in more robust respiratory involvement [47]. Necrosis was evident in non-sensitized mice, indicating that the airway pathology was probably caused by irritancy of the 1% TDI intranasal challenge. Lung inflammation, characteristic of diisocyanate OA in humans, was not evident. A significant contribution of this model was the demonstration that altering the length of sensitization and the cumulative dose resulted in different immunological processes, which may help explain the diversity of symptoms exhibited in humans.

The role of inflammatory mediators in TDI-induced asthma was recently demonstrated by Matheson *et al.* [48] using a murine model employing subcutaneous sensitization and inhalation challenge. Female C57BL/6 mice were sensitized by multiple subcutaneous injections of TDI (20 μl neat TDI, day 1; 20 μl 20% TDI, days 4 and 11) and were then challenged by inhalation with 100 ppb TDI vapor (five times greater than the permissible level for short-term exposures) on days 20, 22 and 24. Non-specific AHR to methacholine was accompanied by mucus metaplasia, inflammation and cytokine expression in the airways. However, inflammation was evident only in the upper airway, with lymphocyte and neutrophil infiltration and inflammatory cytokine expression in the nares and trachea, respectively. Matheson *et al.* [48] found that histopathological changes and inflammatory cell involvement were not evident in athymic mice, supporting the hypothesis that diisocyanate OA is dependent upon specific immunity. Using TNF-α-deficient mice, Matheson *et al.* [49••] identified TNF-α as an integral pro-inflammatory cytokine in disease development. Interestingly, however, mice developed TDI-specific IgG antibodies regardless of TNF-α status, suggesting that IgG antibodies are better markers of exposure than disease in this model. Similarly, diisocyanate-specific IgG generally correlates better with exposure than disease in humans [10•].

In a recently developed model by Herrick *et al.* [50••], female BALB/c mice were sensitized epicutaneously with 0.1 or 1% HDI on days 0 and 7, followed by intranasal challenge with HDI-mouse serum albumin (50 μl at 2 mg/ml) conjugate on days 14, 15, 18 and 19. Mice developed HDI-specific IgG antibodies as well as lymphocyte and eosinophil lung infiltrates. Inflammatory cells from the lung digest produced elevated levels of IL-5, IL-13 and IFN-γ upon restimulation with HDI-mouse serum albumin, indicating a mixed Th1/Th2 immune response, as seen in humans [15,16]. Interestingly, the optimal sensitizing dose for antibody production was higher than that for airway inflammation and cytokine production. This finding is in concordance with that of Matheson *et al.* [49••], suggesting that a different mechanism may dominate for these outcomes. The role of CD4 and CD8 T lymphocytes was also investigated by Herrick and colleagues [51••], who showed that the infiltration of eosinophils into bronchoalveolar lavage fluid was markedly reduced in mice deficient in CD4 T lymphocytes, despite an intact contact hypersensitivity response. This finding is consistent with the attenuated inflammatory response observed in IL-4 and IL-13 knockout mice in the authors' previous work [50••]. In contrast, mice deficient in CD8 T lymphocytes showed a partial reduction in contact hypersensitivity, but no change in the presence of lung inflammatory cells. Together, these findings suggest a dominant role for CD4 T lymphocytes and Th2 immunity in HDI-induced lung inflammation. Herrick *et al.* [51••] also showed that genetics (strain differences) have a strong influence on the development of diisocyanate OA in mice.

Lee and colleagues [52,53••,54••] developed a murine (BALB/c) model of TDI asthma using intranasal sensitization with 3% TDI, using two cycles of five daily instillations with a 3-week rest period between each cycle, and inhalation challenge with 1% TDI one week later. This was the first model to demonstrate *in vivo* AHR after sensitization via the respiratory tract in mice. In addition, significant pathology in the lower airways was evident and was accompanied by a variety of inflammatory mediators. Lee and colleagues [52,54••] demonstrated that the administration of specific inhibitors of two of these mediators, vascular endothelial growth factor (VEGF) or matrix metalloproteinase markedly reduced the disease. A balance between matrix metalloproteinases and their inhibitors is essential for the maintenance of homeostasis of the lung's extracellular matrix, suggesting that disruption of this balance is involved in the pathogenesis of OA.

In order to examine the pathophysiological responses in OA after inhalation exposure, the predominant route of exposure in the workplace, our laboratory has studied two exposure paradigms representative of common workplace exposures [55,56]. In a subchronic exposure model, C57BL/6J mice were sensitized to TDI by inhalation for 6 weeks at the current permissible exposure level (20 ppb) for 4 h a day, 5 days a week, and were challenged 14 days later via inhalation with 20 ppb TDI for 1 h. In the second exposure paradigm, which was intended to mimic an accidental spill, mice received an acute inhalation exposure of 500 ppb TDI for 2 h, and were also challenged 14 days later via inhalation with 20 ppb TDI for 1 h (Matheson *et al.*,

unpublished data) [55,56]. Whereas both groups demonstrated an increase in AHR, the response was considerably more robust after subchronic exposure compared with the acute exposure. Furthermore, although histopathological changes and inflammatory cell infiltration were quite evident in the lungs after subchronic treatment, pathology was primarily confined to the nares and trachea after acute exposure. Total IgG as well as IgG_1 and IgG_{2a} TDI-specific antibodies were elevated in both treatment groups, but total serum IgE levels were increased only in mice that received subchronic TDI exposures (Matheson *et al.*, unpublished data) [55,56]. Although IgE-specific antibodies could not be detected in either group, a direct role for IgE antibodies in the response was observed in the subchronic treatment group, as demonstrated using a modified passive transfer technique [57] in which heat-inactivation of the serum abrogated the response (Matheson *et al.*, unpublished data) [55,56]. Furthermore, evidence of a role for IgE and IgG was demonstrated by the inability of TDI to sensitize FcERIg transgenic mice, which lack the γ chain subunit of the FcεRI, FcγRIII and FcεRI receptors, and thus lack effective IgE and IgG receptors (Matheson *et al.*, unpublished data) [55,56]. Elevated IL-4, IL-5 and IFN-γ expression in the trachea and lungs was also observed in our model after TDI sensitization in both treatment groups, consistent with a mixed Th1/Th2 cytokine profile, as suggested by findings in other animal models [50••,51••] and humans [15,16].

Adoptive transfer experiments were conducted using lymphocytes from TDI-sensitized mice to determine the role of specific immunity in the response to TDI (Matheson *et al.*, unpublished data) [55,56]. AHR was increased in naive mice that received unfractionated lymphocytes, B cells or T cells from sensitized mice. However, AHR persisted only in recipients that received sensitized, unfractionated lymphocytes or T cells, but not B cells (Matheson *et al.*, unpublished data) [55,56]. This probably reflects the temporal nature of the humoral response rather than a lesser contribution by B cells. That isocyanate-induced OA involves a mixed Th1/Th2 phenotype was supported in studies using various transgenic mouse models sensitized subchronically to TDI as described above. For example, IFN-γ-deficient mice demonstrated a reduction in airway inflammation, serum antibody levels and histopathological changes, but to a lesser degree than that observed in IL-4-deficient mice (Matheson *et al.*, unpublished data) [55,56]. In humans with OA, Th2 cells comprise only a small portion of the sensitized T cells in the airways, with most cells representing the Th1 phenotype. The majority of T cell clones derived from the bronchial mucosa of patients with isocyanate-induced asthma present a CD8 phenotype, which produce IFN-γ [19,21••] and IL-5 [19]. In this respect we observed that either CD8 or CD4 deficiency prevented TDI asthma after subchronic treatment (Matheson *et al.*, unpublished data) [55,56].

Conclusion

Controversy surrounds the nature of the antigen, the role of specific antibodies and the role of T-lymphocyte subsets in orchestrating the pathophysiological response leading to diisocyanate OA. Animal models of diisocyanate OA share common features with human disease, although no single model recapitulates the disease in its entirety. Newer murine models of diisocyanate OA have made progress towards a more accurate representation of human exposure and disease. Mouse models have identified a role for both Th1 and Th2 cells as well as CD4 and CD8 T lymphocytes, and evidence now strongly suggests that reaginic antibodies provide a major contribution to disease outcome, indicating the need for better antibody detection systems in humans. Species differences in susceptibility to sensitization and clinical disease outcome strongly support the hypothesis of genetic predisposition to diisocyanate OA and support recent findings in human studies. The continued use of murine models will enable the pursuit of novel findings from patient-oriented studies, such as the role of oxidative stress in diisocyanate OA, and will facilitate an improvement for prevention, therapy and diagnostic interventions.

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