

## Review of the Occupational Exposure to Isocyanates: Mechanisms of Action

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### Abstract

**Polyurethanes are useful polymers in a large variety of technical and consumer products that are generally made from diisocyanates and polyols or similar compounds. Toluene diisocyanate (TDI), 4,4'-methylenediphenyl diisocyanate (MDI) and 1,6'-hexamethylene diisocyanate (HDI) are useful for polyurethane products. Isocyanates are reactive chemicals that can be handled without problems in manufacturing or technical environments. In general, consumers may only have contact with these chemicals on rare occasions. The objective of this study was to review the mechanisms of action of inhalation of isocyanates. This paper describes, in summary, the potential occupational exposure to isocyanates, the chemistry and reactivity of isocyanates, the results from genotoxicity studies, investigative toxicity studies, metabolism and results from epidemiology studies on isocyanate-exposed workers. The overall conclusion is that because humans are not exposed to high levels of respiratory isocyanate particles, concerns over the possible development of lung tumors should not be relevant. There are many mechanisms of action induced by isocyanates, but those entities are unclear. This is because these mechanisms act simultaneously and are complex.**

**Key words:** diisocyanate, immunology, genotoxicity, carcinogenicity, review

### Introduction

Polyurethanes are useful polymers in a large variety of technical and consumer products that are generally made from diisocyanates and polyols or similar compounds. Toluene diisocyanate (TDI), 4,4'-methylenediphenyl diisocyanate (MDI) and 1,6'-hexamethylene diisocyanate (HDI) are useful for polyurethane products such as foam plastic products and polyurethane resin. Isocyanates are reactive chemicals that can be handled without problems in manufacturing or technical environments.

Isocyanates are characterized by the  $N=C=O$  group which contains two double bonds and exhibits strong chemical reactivity. The most relevant diisocyanates (toluene diisocyanate: TDI; 4,4'-methylenediphenyl diisocyanate: MDI; 1,6'-hexamethylene diisocyanate: HDI) are known as the main causes of sensitivity by low molecular-weight chemicals, and as moderate irritants. Those mechanisms are not clear, but they probably act as haptens. It is known that isocyanates have cross-reactivity<sup>1)</sup>. Here, we review the mechanisms of action of these diisocyanates<sup>2)</sup>.

In many countries, including Japan, there are many examples

where chemicals induce occupational symptoms that produce new problems after their sensitization. These problems need to be identified. In other words, allergic health disorders following even a little exposure to such chemicals plays a major role in problems of working health.

### Mechanisms of action after exposure

#### *Animal studies*

Animal inhalation studies with <sup>14</sup>C-labeled MDI and TDI demonstrated that these chemicals are mainly absorbed in the upper airways<sup>3,4)</sup>. They are found in the epithelium and at the subepithelial level from the nose down to the terminal bronchioles. Using good guinea-pig models displaying both immunologic and respiratory hypersensitivity to TDI, TDI-adducts in respiratory tissues can be detected with specific rabbit antiserum prepared to TDI-keyhole limpet hemocyanin<sup>5,6)</sup>. In the previous studies<sup>7)</sup>, at least five TDI-adducted proteins were detected in the bronchoalveolar lavage fluid (BALF) of TDI-exposed guinea-pigs. The most prominent adducted protein was serum albumin<sup>8)</sup>. In addition to airway tissues, blood is also a primary target of inhaled isocyanates. Carbamoylated TDI-hemoglobin adducts as well as an aromatic nitroso adduct of hemoglobin in erythrocytes of guinea-pigs were identified after TDI exposure<sup>9,10)</sup>. A study by Kennedy et al.<sup>4)</sup> in a rat <sup>14</sup>C-TDI exposure model showed that, in addition to the airways and the gastrointestinal system, blood had the highest level of detectable quantities of radioactivity, which increased with exposure concentration. The radioactivity concentration in the

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bloodstream after exposure was linear with respect to dose and was between 3 and 10% of the inhaled uptake. The majority (74–87%) of the <sup>14</sup>C-labeled products associated with the blood was recovered in plasma, and 97–100% of this existed in the form of biomolecular conjugates. Acetylated MDI-hemoglobin adducts were also detected in rats exposed to MDI<sup>11</sup>. Incidentally, it is known that TDI can be easily hydrolyzed, and changed to toluene diamine (TDA), although Sabbioni et al.<sup>11</sup> also concluded that conjugation is the predominant reaction after TDI exposure in rats, and that TDA is not a primary *in vivo* reaction product under the conditions they tested. In rats, hemoglobin (Hb) adducts were found to correlate with the administered dose of either MDA (4,4'-methylene dianiline) or MDI<sup>11–13</sup>.

#### Human studies

In human studies, following inhalation of isocyanates, isocyanate-protein adducts can be detected. In one case report<sup>14</sup>, a challenge test in the workplace was performed, and it was suggested that isocyanate-adducts exist in pulmonary tissue after HDI exposure. Therefore, adducts with blood proteins are suitable markers for exposure and target-dose estimations<sup>14</sup>. According to other studies<sup>10,15</sup>, albumin is the major receptor molecule for 2,4- and 2,6-TDI in blood plasma of exposed workers. These diisocyanates covalently bind to albumin. Thus, diisocyanate could be regarded as a homofunctional agent capable of cross-linking different polypeptide chains by intra- or intermolecular linkages. A study on serum protein alterations following inhalation exposure to TDI vapors demonstrated that human prealbumins undergo rapid changes in patients after TDI exposure<sup>16</sup>.

TDI-adduct formation with epithelial proteins suggests epithelial injury and implies possible alteration in airway permeability, cell signaling, and increased sensitivity to airway receptors. Epithelial cells can produce cytokines and various pro-inflammatory mediators that contribute to airway inflammation. The most major finding of isocyanate-induced asthma is that, airway inflammation is observed primarily with DAR (Dual asthmatic response) and LAR (Late asthmatic response)<sup>17,18</sup>. In the mucosa or submucosa of asthma patients induced by isocyanates, histological findings such as mucosal hypertrophy were similar, but inflammatory cells such as CD25-positive cells or eosinophils increased. An increase in mast cells could be seen only in the mucosa<sup>17</sup>. These inflammatory cells decreased after cessation of exposure<sup>18</sup>.

High concentrations of diisocyanates directly lead to lesions of the lung or airway and even to disruption of the mucus layer, which is the major site of mast cell- and eosinophil-related mucosal inflammatory responses<sup>2</sup>.

### Genotoxic, mutagenic and carcinogenic effects

#### Genotoxicity

Isocyanates (especially in diisocyanates) and some of their metabolites, for example, diamines, can form macromolecular adducts not only with proteins such as albumin or hemoglobin but also with DNA<sup>19</sup>. Marczynski et al.<sup>20,21</sup> showed *in vivo* inhalation of MDI or *in vitro* incubation with TDI induced double-strand breaks (DSB) in white blood cells of exposed workers or in isolated blood cells. Since purified DNA treated with TDI in buffer did not induce DNA fragmentation, it has to be assumed that DNA damage was due to diisocyanates metabolites obtained after bioformation. It has to be considered that DSB and cross-

links on DNA lead to chromosomal damage and mutagenic effects. *In vitro* results showed that TDI can induce degradation of mitochondrial DNA and large DNA fragments, which results from apoptosis, into small DNA fragments<sup>22</sup>. In investigation of the induction of DNA DSB by MDI in cultured human lung epithelial cells (A549), the observed DSB were the consequence of extragenomic damage in the course of cell death rather than of an interaction with DNA<sup>23</sup>. MDI produced only irregular clumping of chromatin (72 hr point), and induced smaller DNA fragments in a time-dependent manner. Thus, it is suggested that DSB observed in cells treated with MDI are unlikely to be the result of DNA cross-link formation<sup>23</sup>. In addition, in the study of Czuppon et al.<sup>24</sup>, induction of anti-dsDNA autoantibodies were measured at increased concentrations in sera of workers occupationally exposed to diisocyanates.

Recently, the possibility has been raised of prenatal toxicity of inhaled isocyanates. Therefore, in pregnant wister rats, an inhalation study using polymeric MDI was performed<sup>25</sup>. In that study, exposure to 12 mg/m<sup>3</sup> of respirable polymeric MDI aerosol resulted in maternal toxicity, including mortality of 2 of 25 dams. However, there was no evidence of maternal or developmental toxicity at 1 or 4 mg/m<sup>3</sup>. The no-observed adverse effect level (NOAEL) for maternal and developmental toxicity was therefore 4 mg/m<sup>3</sup>. There were no treatment-related teratogenic effects at any concentration evaluated<sup>25</sup>.

The diamine produced by hydrolyzed TDI and MDI, which are TDA and MDA, respectively, induced cancer in rats<sup>26–28</sup>. Therefore, the metabolic fate of TDI *in vivo*, particularly its hydrolytic conversion to TDA, is important with regard to the assessment of risk for cancer. TDI and MDI were also found to induce chromosome aberrations and sister-chromatid exchange (SCE) after a 24 hr treatment in the absence of additional metabolic activation in human cultured lymphocytes<sup>29</sup>. SCE was also induced in Chinese hamster ovary (CHO) cells by TDI<sup>30</sup>.

#### Mutagenicity

TDI<sup>31</sup> and MDI<sup>32</sup> are mutagenic. However, the findings<sup>33–36</sup>, especially using Ames test<sup>33–34,36</sup>, need consideration. While many studies found positive Ames tests with MDI and TDI, these used DMSO (dimethylsulfoxid) or other solvents to disperse the diisocyanates into the medium. It was recently shown that MDI and TDI rapidly degrade in aqueous systems with DMSO, and also in the presence of the Ames test mixture<sup>37</sup>, and generation of TDA in the system probably accounts for the positive result. Due to the low water solubility of these diisocyanates, and their protein reactivity, it is not possible to properly test them in *in vitro* systems. Although no activity in the mutation tests was detected with any MDI isomer in the absence of S9, S9 irrespective of whether DMSO or ethylenglycol dimethylether (EGDE) were used as vehicles, no mutagenicity found when EGDE was used in the presence of S9. In contrast, MDI is stable in EGDE and no mutagenic activity was detectable in microbial systems<sup>38</sup>. They also clearly demonstrated that no mutagenic activity was detectable under conditions where MDA was not produced.

#### Carcinogenicity

The class of carcinogenicity of MDI is 3<sup>32</sup> according to IARC, although HDI is not yet classified. However, TDI is carcinogenic in animals (the class of carcinogenicity is 2B)<sup>31</sup>, but it has not been clarified whether occupational exposure to such chemicals

is associated with an increased risk of cancer<sup>39)</sup> in humans. This is because positive results using experimental animals are only in studies of administration by gavage. No treatment-related tumor was observed after exposure of mice or rats to commercial TDI by inhalation<sup>31)</sup>. Furthermore, there is no known case of occupational cancer by TDI exposure. For this reason, it is interesting that few epidemiological cancer studies did not lead to clear results<sup>40-42)</sup>.

### Immunological mechanisms and clinical responses

Cell-mediated immune responses appear to play an important role in the early pathogenesis of symptoms induced by isocyanates. Activation of the specific immune response requires the uptake of foreign antigens and their presentation to lymphocytes by macrophages. The best antigen-presenting cells (APCs) in the lungs are dendritic cells, forming a network between the epithelial cells<sup>43)</sup>. Effective APCs must endocytose potential antigens, proteolytically digest them, and then associate the resulting peptides with MHC (major histocompatibility complex) molecules before transporting the MHC-antigen complex to the cell surface. In the case of isocyanate, as well as most of the high molecular weight allergens, only scant findings on antigen processing and presentation are available<sup>44,45)</sup>. It is necessary to consider that the extent of exposure to the allergen and the way in which that allergen is processed and presented to T lymphocytes can have important implications for the quality of the immune response induced, and whether isocyanate activates monocytes or macrophages<sup>46,47)</sup>. For successful T cell activation, the interaction between the MHC-peptide complex and T cell receptor (TCR) on T cells and the expression of accessory molecules on APCs are required. Determination of the T cell receptor repertoire and HLA association may be useful for the evaluation of disease susceptibility by these two parameters<sup>48,49)</sup>. Bernstein et al.<sup>50)</sup> reported evidence that the involvement of the TCR V $\beta$  repertoire expression increased the expression V $\beta$ 1 and V $\beta$ 5 after *in vitro* stimulation in patients who were exposed to diisocyanate. Bignon et al.<sup>51)</sup>, Balboni et al.<sup>52)</sup> and Mapp et al.<sup>53)</sup> studied the HLA class II alleles' contribution to susceptibility or resistance to isocyanate-induced asthma in exposed workers. They reported that allele DQB1\*0503 and the allele combination DQB1\*0201/0301 were associated with susceptibility to the disease. In contrast, alleles DQB1\*0501 and the DQA1\*0101/DQB1\*0501/DR1 haplotype were suggested to confer protection to exposed but healthy control subjects. The authors suggested that immune mechanisms are involved in isocyanate-induced asthma and that specific genetic factors may increase or decrease the risk of disease development in exposed workers. These findings were, however, neither confirmed by Bernstein et al.<sup>54)</sup>, nor Rihs et al.<sup>55)</sup>.

When CD4<sup>+</sup> T cells (Th0) are activated by stimulation of their antigen receptors (via MHC class II-peptide complex) and accessory ligands, they begin to produce a number of soluble factors (cytokines) which influence other cells<sup>56)</sup>. In addition, the T cells proliferate, expanding the pool of antigen specific T cells leading to enhanced responsiveness and immunological memory. Differentiation of Th0 to the Th1 or Th2 subtypes characterized by their typical cytokine pattern depends on the nature of the antigen (e.g., allergen or mycobacteria), cooperating cells (e.g., mast cells or NK cells), and cytokines (IL-12 or IL-4). Allergenic antigens and an IL-4-rich environment (provided by IL-4 secreted from mast cells) encourage the development of the Th2 phenotype. Th2

cells are characterized by an enhanced production of IL-4, IL-5, IL-3, and GM-CSF but little or not by IFN $\gamma$ <sup>57)</sup>. The Th2 subtype is associated with IgE and eosinophilia and hence with allergy. In experimental studies, in serum of mice exposed to diisocyanates by inhalation not only total IgE but also specific IgE, IgG and IL-2, 4, 5, and IFN $\gamma$  were increased<sup>58-61)</sup>. Inflammatory cell infiltrates in bronchial biopsies of patients suffering from isocyanate induced asthma include mast cells, eosinophils, and activated lymphocytes (bearing the IL-2 receptor (CD25))<sup>62,63)</sup>. The presence of activated lymphocytes and eosinophils in bronchial biopsies suggest that a T lymphocyte eosinophil interaction may be important in asthma of different origin, a hypothesis further supported by the finding of the cells expressing IL-5 messenger RNA in bronchial biopsies of asthmatics. IL-5 is in fact the most important eosinophil regulating cytokine and its concentration in the airway mucosa of asthmatics correlates with activation markers of T lymphocytes and eosinophils<sup>64,65)</sup>. In further studies, Fabbri et al.<sup>66)</sup> and Maestrelli et al.<sup>64,65)</sup> determined whether specific *in vivo* stimulation of asthmatics sensitized by TDI induced activation of T lymphocytes in bronchial mucosa and they characterized the phenotypes and cytokine secretion profile. For this purpose, they generated T cell clones from endobronchial biopsies. Most of the clones exhibited the CD8 phenotype<sup>64,67)</sup>. All of these CD8 clones produced IFN $\gamma$  and 44% of these produced IL-5, but only a small amount also secreted IL-4. Additional results<sup>64,67)</sup> suggest that the exposure to TDI induces a transient increase in the number of cells storing IL-4 and IL-5 in the bronchial mucosa<sup>67)</sup>.

Immune responses to isocyanates may induce several isocyanate-induced diseases, and cell or antibody-mediated responses. The production of specific IgE and IgG antibodies<sup>68,69)</sup> and the predominance of CD8 cells are features of different symptoms. Therefore, symptomatic workers exposed to isocyanates are mostly diagnosed as asthma at first.

In addition, there are several isolated case reports describing isocyanate-induced hypersensitivity pneumonitis (HP). HP is defined as airspace and wall alveolitis, or diffuse lung dysfunction with epithelioid cell granuloma, or inflammatory incarnant interstitial pneumonia caused by repeated inhalation of occupational factor especially organic materials such as isocyanates<sup>70)</sup>. Isocyanate-HP is granulomatous inflammatory reaction in terminal airways, alveoli and the surrounding interstitium. Extensive information on the pathology of this disease was obtained by open lung biopsies and by analysis of BALF. According to the pathogenesis, there are several lines of evidence supporting the involvement of both type III immunity (IgG-dependent), which may lead to the formation of immune complexes and the activation of the complement cascade, and type IV immunity (cellular) mediated by antigen-responsive T cells with conjugates of isocyanate and human serum albumin. They can be detected in BALF<sup>71)</sup> or peripheral blood<sup>72)</sup>. Although the individual pathogenic role of these responses is not clear, it can be assumed that diagnosis parameters, such as specific IgG antibodies and antigen-specific lymphocyte reactions, are operative in this chronic interstitial lung disease<sup>73)</sup>. Since isocyanate-HP is one of the well-known diseases induced by isocyanate, we also discuss the mechanisms of isocyanate-HP in another article in detail<sup>74)</sup>.

There are also various other diseases such as conjunctivitis, dermatitis, and rhinitis<sup>75-77)</sup>. Dermatitis is not lethal, which is different from asthma or HP. The patient thinks that it is only a rash, so it is probably overlooked whether the source is isocyan-

ates or not. In Japan, there were two case reports of dermatitis caused by TDI (gas, fluid)<sup>78,79</sup>. Since knowledge about isocyanates is widespread, people do not generally take unsafe actions such as working with ungloved hands. In dermatitis or urticaria, isocyanates act as irritants and patients showed type IV allergy. On the other hand, asthma shows type I allergy and HP shows both type III and type IV allergies. Therefore, their mechanisms of actions are suggested to be little different from that of asthma or HP, from the immunological point of view. Even if findings are not severe, such diseases appear to be early stages of severe diseases such as asthma or HP. For example, car painters develop dermatitis caused by paints that include isocyanates. Dermatitis was recently recognised to be a risk index for isocyanate asthma<sup>80</sup>.

## Conclusions

The analysis of BALF cells or TBLB cells, and soluble mediators (cytokine) from workers using isocyanates and the correlation of inflammation with clinical parameters have provided new insights into the pathogenesis of diseases induced by isocyanates. Some features such as cells and cytokine proliferations are comparatively similar to other occupational allergic inflammations, and other features are different. For example, in non-IgE-mediated asthma, bronchial eosinophilia appears to be directly caused via the local production of IL-5 (Th2 type) attracting, activating, and increasing the survival of eosinophils. Inflammatory cells, (especially eosinophils) and T cells (CD4 and especially CD8 cells) and

their interaction and cooperation appear to be important. It has to be considered that the degrees of exposure to allergen processed and presented to T cells may have important implications for the quality of the immune response induced. It is important to evaluate the exposure-response relations between diisocyanates and the induction of sensitization and elicitation of the relevant immunological processes.

In this paper, the potential exposure to isocyanates, the chemistry and reactivity of isocyanates, results from genotoxicity, investigative toxicity studies, metabolism and results from epidemiological studies on isocyanates exposed workers are described. The overall conclusion is that because humans are not exposed to high levels of respiratory isocyanate particles, concerns over the possible development of lung tumors should not be relevant. There are many mechanisms of action induced by isocyanates, but those entities are unclear. This is because these mechanisms act simultaneously and are complex.

In general, basic research on immunology, molecular, and cellular biology together with detailed clinical examinations have help clarify the future of worker health.

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