Toluene Diisocyanate Colocalizes with Tubulin on Cilia of Differentiated Human Airway Epithelial Cells

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Toluene diisocyanate (TDI), a highly reactive industrial chemical with widespread use in the manufacture of polyurethane and plastics, is the leading cause of occupational asthma associated with chemical exposure. We report the effects of TDI vapor (20, 100, 500, 1000 ppb) in vitro on differentiated human bronchial epithelial cells. Increased mucus was observed by electron microscopy at all TDI concentrations. Cytotoxicity, as evidenced by cell pyknosis and DNA fragmentation, was detected following a 30-min exposure to TDI concentrations of 100 ppb or higher. At 1000 ppb, transepithelial resistance was lost. Using confocal microscopy and double staining, TDI was found colocalized with ciliary tubulin in cultures that had been exposed to 20 and 100 ppb. These findings are the first to identify TDI binding to human pulmonary epithelial cells; ciliary tubulin. The morphology and integrity of the cells following TDI exposure were also studied. The results will assist in elucidating the nature of TDI-modified molecules of airway epithelial cells and their role in the pathogenesis of TDI airway disease.

MATERIALS AND METHODS

Chemicals. Toluene diisocyanate (TDI, 4:1 molar mixture of 2,4 and 2,6 isomers) was provided by Bayer, USA (Pittsburgh, PA). Human bronchial epithelial (HBE) cell culture. Non-carcinogenic, surgical specimens were obtained from lung transplant recipients. Tracheo-bronchial cells were isolated as previously described (Mette et al., 1993). Dr. Joseph M. Pilewski, University of Pittsburgh Medical Center provided these primary HBE cell cultures. Briefly, for culture, cells were passaged twice in keratinocyte-serum-free medium (K-SFM, Gibco, Gaithersburg, MD) containing epidermal growth factor (EGF, 5 ng/ml, Gibco), bovine pituitary extract (BPE, 50 μg/ml, Gibco), Fungizone (1%, Gibco), penicillin (100 U/ml, Gibco), streptomycin (100 μg/ml, Gibco) and L-glutamine (2 mM, Gibco). Upon confluence, HBE were trypsinized (0.05% w/EDTA, Gibco) and seeded 1:1 (surface area) on 12-or 24-mm (12- and 6-well plates respectively) polyester albumin, in the lavage fluid of guinea pigs following inhalation exposure (Jin et al., 1993). It was also associated with inflammatory cells (Jin et al., 1993) and appeared at the apical surface of epithelial cells in the upper respiratory tract (Ebino et al., 1998; Karol et al., 1997). Molecular characterization of the adducted biomolecules is expected to result in a better understanding of the pathogenesis of TDI respiratory diseases.

The upper airways of the human respiratory tract are a mosaic of lining and secretory epithelial cells (Weibel, 1985). In response to environmental agents, these cells release cytokines and other soluble factors that modulate airway smooth muscle contraction (Arnold et al., 1994; Egilmez and Ilhan, 1992). Methods exist for the growth and differentiation of human bronchial epithelial (HBE) cells isolated from surgical biopsies (Mette et al., 1993). Importantly, HBE cells cultured at an air/liquid interface express critical differentiation characteristics, such as mucus and cilia (de Jong et al., 1994).

The present study sought to identify the interactions of TDI with primary, differentiated human airway epithelial cells following exposure to concentrations of the chemical vapor that included that maximally allowable in the workplace (20 ppb). The morphology and integrity of the cells following TDI exposure were also studied. The results will assist in elucidating the nature of TDI-modified molecules of airway epithelial cells and their role in the pathogenesis of TDI airway disease.
Transwell™ filters (Corning-Costar, Corning, N.Y.) in K-SFM, fed with 1-ml medium inside and 1.5-ml outside the transwell. When the cells were confluent, as assessed microscopically, the inside medium was removed and the outside medium was replaced with 1:1 D-MEM/F-12 (Gibco) containing Fungizone (1%, Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml, Gibco), L-glutamine (2 mM Gibco), and ULTROSER G (2%, BioSepra S. A., France). Medium was changed on alternate days. HBE cells were incubated 14–21 days at the air/liquid interface (ALI) prior to TDI exposure.

**Exposure of HBE to TDI.** Filters containing the ALI-cultured HBE cells were cut from the Transwell™, placed on a stainless steel sieve in a 28-cm glass petri dish. ALI medium (0.75 ml) was added to wet the basal surface of the filter. The open petri dish was placed in a glass chamber, and cells were exposed for 30 min to atmospheres of TDI (0.02–1.0 ppm) generated by bubbling dried air through 5 ml TDI held in a glass impinger (Jin et al., 1993). Chamber humidity was maintained at 50% using an external source of water vapor. The atmosphere was monitored using a real time Autostep™ gas analyzer equipped with an isocyanate trapping tape (Bacharach, Inc., Pittsburgh, PA). For concentrations greater than 100 ppm, atmospheres were analyzed using an in-line filter and employing HPLC after derivitization with p-nitrobenzoylpropylamine (Jin et al., 1993). Sham exposures were performed in an identical manner using water in place of TDI in the impinger. Pilot studies indicated that 30-min exposure did not dehydrate the cell monolayer.

**Flow cytometry.** Propidium iodide intercalation into DNA was quantified to estimate cytotoxicity. Following exposure to TDI or air, cells were removed from triplicate filters (24-mm) by scraping with a rubber policeman. Each filter contains approximately 1 × 10^6 cells and represents one sample. Cells and subsequent washes (totaling 1 ml D-PBS) were transferred to a 1.5-ml tube and centrifuged for 5 min at 4°C, 2000 rpm. Cells were resuspended in 200 μl HBBS, fixed in 70% ethanol (4°C for ≥30 min, usually overnight) then centrifuged. The pellet was resuspended in 0.9 ml HBBS containing RNase A (100 μg/ml, Boehringer Mannheim Corp., Indianapolis, IN) and incubated for 10 min at 37°C. Propidium iodide (0.1 ml, 1mg/ml, Sigma, St. Louis, MO) was added, and the cells were stored at 4°C in the dark until analyzed. DNA content was determined from forward and orthogonal light scatter, and peak and area red fluorescence (Becton-Dickinson FACScan flow cytometer equipped with a 488 nm AR laser). Cell populations were quantified from a standard count of 10,000 cells using the Lysis II program.

**Microscopic evaluation of HBE cells.** For both confocal and electron microscopy, filters (12-mm) containing HBE cells were fixed immediately following TDI exposure. To detect TDI adducts using confocal microscopy, filters were placed in 10% buffered formalin and held at 4°C until processed. Cells were first stained with the TDI antibody. Filters containing the fixed epithelial cells were incubated with antisera for 16 h at 4°C. TDI adducts were detected using an antiserum (1:300) produced to TDI-keyhole limpet hemocyanin (Jin et al., 1993). Washing sections in the appropriate serum blocked nonspecific binding. Cilia were localized using a monoclonal antibody (1:400) against β-tubulin (Sigma, St. Louis, MO). Incubation was carried out at room temperature for 30 min. FITC-(for tubulin) and Cy5-(for TDI) labeled secondary antibodies (1:200) were used to visualize the primary antibodies. Controls consisted of incubation in the absence of primary and/or secondary antibodies. Nonspecific binding was not detected in any of the control specimens. Epithelial cell nuclei were visualized using YOYO-1 (Molecular Probes, Eugene, OR). Slides were treated with RNase prior to staining with YOYO. Images were obtained using a Leica TCS confocal microscope equipped with a Kr/Ar laser and a Leica IRBE inverted microscope. All images were taken with a 100× oil, NA 1.4 lens. Distance between optical sections was approximately 1 μm. Overlays of sections were created using Adobe Photoshop.

For electron microscopy, cells were grown on filters (12 mm) and fixed in 2.5% glutaraldehyde in PBS (8 gm/l NaCl, 0.2 gm/l KCl, 1.15 gm/l Na2HPO4, 7H2O, 0.2 gm/l KH2PO4, pH 7.4) overnight at 4°C. Monolayers were then washed in PBS 3 times and post-fixed 1 h in aqueous 1% osmium tetroxide, 1% FeCl3. Cells were washed 3 times in PBS, dehydrated through a 30–100% ethanol series and several changes of Polybed 812 embedding resin (Polysciences, Warrington, PA). Filters were cut into 1 × 2 mm2 strips and embedded in Polybed 812. Blocks were cut overnight at 37°C, and for 2 days at 65°C. Ultrathin cross sections (60 nm) were obtained on a Riechert Ultracut E microtome, collected on formvar-coated grids, post-stained in 4% uranyl acetate for 10 min, and 1% lead citrate for 7 min. Sections were viewed on a JEOL JEM 1210 or 100CX transmission electron microscope at 80 KV.

**Membrane resistance measurements.** To evaluate the integrity of the HBE monolayers following TDI exposure, transepithelial resistance was measured. Cells cultured on 24-mm filters were exposed to TDI and maintained at 37°C for approximately 2 h prior to analysis. The monolayer was voltage-clamped to zero by an external current passing circuit to correct for fluid resistance, and the resulting Isc was recorded continuously using Acquire 6600 software (Gould Instruments Systems, Inc., Valley View, OH) on a personal computer. Monolayers were pulsed with a 2mV bipolar pulse.

**RESULTS**

To establish appropriate TDI exposure levels, cells were examined for viability and cytotoxicity. Following TDI exposure, cell viability was assessed by propidium iodide, single color, flow-cytometric analysis. The percent of non-viable cells increased in a dose-dependent manner that reached significance (p < 0.05) at 100 ppb TDI (Table 1).

**TABLE 1**

<table>
<thead>
<tr>
<th>TDI concentration (ppb)</th>
<th>% Dead</th>
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<tbody>
<tr>
<td>0</td>
<td>17.1 ± 3.7</td>
</tr>
<tr>
<td>20</td>
<td>12.8 ± 8.4</td>
</tr>
<tr>
<td>50</td>
<td>20.8 ± 11.2</td>
</tr>
<tr>
<td>100</td>
<td>23.3 ± 8.2*</td>
</tr>
</tbody>
</table>

Note. TDI-induced cytotoxicity following 30-min exposure of cells to the chemical. 0 ppb represents 30-min sham exposure created by passing air through an impinger that contained water in place of TDI. Percent dead represents the fraction of 10,000 cells that contain fragmented DNA as analyzed by flow cytometry. Results are expressed as mean ± SEM from triplicate determinations of a representative experiment.

*p < 0.05 vs. sham-exposed (0 ppb) cells.

HBE monolayer integrity was determined from measurement of transepithelial resistance. Membrane resistance was unchanged following a 30-min exposure to TDI concentrations of 100 ppb or lower, indicating that, at these concentrations, tight junctions and apical membranes remained intact. Complete loss of resistance was observed following 30 min at 1.0 ppm TDI, a concentration that caused cell death (Table 2).

Based on these data we lowered the maximum concentration of 100 ppb or lower, indicating that, at these concentrations, tight junctions and apical membranes remained intact. Complete loss of resistance was observed following 30 min at 1.0 ppm TDI, a concentration that caused cell death (Table 2). Based on these data we lowered the maximum concentration used to 500 ppb.

Evaluation of the TDI-induced ultrastructural changes in HBE cells was assessed by transmission electron microscopy. Sham-exposed HBE cells displayed cilia, mucus, numerous mitochondria, highly interdigitating cell contacts, and intact
tight and gap junctions (Figs. 1A and 2A). HBE cells exposed to 20 ppb TDI exhibited features consistent with the sham-exposed cells (Fig. 1B). HBE cells exposed to 100–500 ppb TDI vapor demonstrated increasing pyknosis. This was further made evident by the reduced volume of intracellular matrix and the loss of ultrastructure, i.e., organelles and intercellular junctions in the 100–500 ppb samples (Figs. 1C and 1D, 2C and 2D). Apical secretions and glycogen aggregates were also more evident in cultures exposed to TDI (Figs. 1B–1D, 2B–2D). The higher magnification ($\times 31,200$) further demonstrates the mucous adherence, glycogen accumulation, and disorganization of the tight and gap junctions. Few tight junctions were apparent in cells exposed to 500 ppb (Fig. 2D).

### TABLE 2
Transepithelial Resistance of HBE Cells following TDI Exposure

<table>
<thead>
<tr>
<th>TDI concentration (ppb)</th>
<th>Ohm (Ω cm$^2$)</th>
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<tbody>
<tr>
<td>0</td>
<td>762 ± 127</td>
</tr>
<tr>
<td>10</td>
<td>892 ± 161</td>
</tr>
<tr>
<td>100</td>
<td>851 ± 82</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
</tr>
</tbody>
</table>

*Note.* TDI-induced monolayer resistance alteration following 30-min exposure to the chemical. 0 ppb presents 30-min sham exposure created by passing air through an impinger that contained water in place of TDI. Results are expressed as mean ± SEM from triplicate determinations of a representative experiment.

**FIG. 1.** Electron micrographs of HBE cells following 30-min exposure to air or TDI vapor. Magnification $\times 8400$. (A) Sham-exposed preparation. Note the prominent cilia. (B) HBE cells exposed to 20 ppb TDI. Cilia and cell integrity is indistinguishable from sham-exposed cells. Increased mucus is seen at the cell surface. (C) HBE exposed to 100 ppb TDI. Cell pyknosis is apparent. Increased accumulation of glycogen (dark granules) and mucus is seen. (D) HBE exposed to 500 ppb TDI. Cell pyknosis is severe; cellular ultrastructure is obliterated. Scale bar, 500 nm.
TDI Localization in HBE Cells

Immunostaining of HBE cells with anti-TDI antibody indicated the cellular localization of TDI. In Figure 3, TDI staining (red) was observed only in cultures that received TDI exposure (Figs. 3C–3F). Nuclei stained with the nucleic acid stain YOYO appeared blue (Figs. 3A, 3C, and 3E). From the pattern of staining, TDI appeared to be located predominately on the cilia (Figs. 3C–3F).

Further evidence that TDI was localized to the cilia was obtained from optical sections and double staining. TDI was predominantly seen in sections that were above the plane of the nuclei (Figs. 3D and 3F). A dose-dependent increase in TDI staining was apparent between the 20- and 100-ppb exposures. While the 100-ppb exposure produced uniform staining on most cilia, 20-ppb exposure produced more sporadic staining for TDI (Figs. 3C with 3E, and 3D with 3F).

Cilia were visualized using an antibody directed against tubulin. Localization of TDI to the cilia is apparent from the orange to yellow color (compare Fig. 3C–3F), indicating the colocalization of TDI with $\beta$-tubulin.

DISCUSSION

Several low-molecular-weight (LMW) chemicals, such as disocyanates, are recognized for their ability to cause occupational asthma (Karol et al., 1996; Mapp et al., 1994). Unfor-
Flow cytometry was used to estimate the TDI-induced cytotoxicity to the ALI cultures. We noted some cell death in the sham-exposed cultures and attribute this to the necessary scraping of cells from the filters and dissociation of cell aggregates. Nonetheless, a significant elevation in cytotoxicity was observed in cultures exposed to 100 ppb TDI compared with the sham-exposed cells.

The effect of TDI on cell membrane integrity was examined using transmission electron microscopy (TEM) and transepithelial resistance. Sham-exposed cells demonstrated extensive interdigitations and numerous tight junctions. In cells exposed to TDI concentrations at or above 100 ppb, ultrastructural alterations included glycogen and mucus accumulation. At 500 ppb, disorganized tight and gap junctions were visible. Lower concentrations did not alter the integrity of the membrane or the tight junctions, as measured by resistance. Since measurements were made several hours following the TDI exposure, recovery may account for the tight monolayers observed at low TDI concentrations (Cunningham et al., 1993).

Tubulin immunolabelling is routinely used to detect ciliated cells of the airway epithelium (Chapelin et al., 1996). Using double labeling, we observed TDI to be associated with cilia and colocalized with tubulin on the airway cells. Previously, it was demonstrated that TDI rapidly formed adducts with the sulphydryl moiety of glutathione (Day et al., 1997). Tubulin, a subunit protein of ciliary microtubules, integrates ciliary motion with cellular function via the cytoskeleton (Wheatley et al., 1996). It possesses numerous sulphydryl moieties.

The secretory and ciliated cells of the upper respiratory airway provide the primary defense against airborne xenobiotics (Pavia, 1984). Mucociliary action continuously clears the luminal surface of the airway, thus decreasing the duration of xenobiotic exposure. Deciliation or impairment of mucociliary activity by inhaled agents such as TDI may increase pulmonary exposure and contribute to the pathophysiology.

The apparent TDI-induced increase in mucus adhesion would reduce airflow clearance. This change may be a result of TDI-induced alterations of mucus, ion transport at the apical membrane, or ciliary motion. Hypersecretion of mucus has been shown to occur in response to oxidants and may represent a fundamental pathogenesis for a variety of pulmonary disorders (Wright et al., 1996). The first documented asthma death, induced in a TDI-sensitized individual by isocyanate exposure, was associated with mucous plugging of the bronchi, suggesting that TDI asthma is associated with airflow hypersecretion (Fabbri et al., 1988). The increase in glycerogen aggregates noted in TDI-exposed cells suggests that TDI alters energy...

![FIG. 3. TDI localization in differentiated HBE cells. Binding of TDI to HBE cells was analyzed in cultures exposed to air (A and B), 20 ppb TDI (C and D) and 100 ppb TDI (E and F). Pictures in the left column are composite overlays of confocal images taken through the entire cellular layer. Pictures in the right column are single optical sections taken above the plane of the nuclei. Nuclei are represented in blue, tubulin in green, and TDI in red. Colocalization of tubulin and TDI appears orange to yellow (E and F). Colocalized tubulin and TDI staining was seen predominantly in optical sections that were above the plane of the nuclei. TDI staining is more intense and uniform in the100 ppb cultures compared to the 20 ppb exposures. No TDI staining was observed in the sham-exposed controls.](https://example.com/figure3.png)
producing oxidative metabolism, which would further impair mucociliary activity.

TDI-adducted tubulin may contribute to the etiology of asthma by impairing ciliary motion and affecting cytoskeletal signaling. Because of its interaction with cytoskeletal proteins, the perturbation of ciliary tubulin can alter membrane receptors or G proteins (Ravindra et al., 1997) and MUC gene expression. Cytoskeletal alterations modify cell shape and facilitate cell migration and invasion (Kheradmand et al., 1998). These changes would allow greater access of TDI-modified molecules and cells to the underlying tissue and favor stimulation of immune cells. Furthermore, ciliated epithelial cells contain endopeptidases that regulate airway neutopeptide activity and ultimately airway tone (Choi et al., 1990). Epithelial bound TDI may interfere with these enzymes, prevent the degradation of bioactive peptides such as substance P, and potentiate airway hyperreactivity (Gagnaire et al., 1997). Mucus hypersecretion, activation of lymphocytes, and airway hyperreactivity are recognized hallmarks of TDI asthma.

To our knowledge this is the first report of TDI binding to human pulmonary epithelial cells and causing morphological and chemical alterations. Binding was observed with the lowest TDI concentration used, 20 ppb. This concentration is the current permissible exposure level (PEL) for industrial workers in the U.S. The results indicate a TDI-induced series of changes in epithelial cells that would have profound effects on cell function. Further biochemical, and functional analyses are required to understand the physiological consequences of the changes and the relevance of adducts to isocyanate sensitization.

ACKNOWLEDGMENTS

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REFERENCES


