

Identification of Human Lung and Skin Proteins Conjugated with Hexamethylene Diisocyanate *In Vitro* and *In Vivo*

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Diisocyanates are asthma-causing chemicals used in the commercial production of polyurethane. We have previously shown that human lung epithelial cell proteins can become conjugated with hexamethylene diisocyanate (HDI) and may be biologically important in diisocyanate-induced asthma. The objective of this study was to identify specific human lung and skin proteins that become conjugated with diisocyanate after *in vitro* and *in vivo* exposure. Following *in vitro* exposure of human airway epithelial cells (A549), keratin 18, the 78-kD glucose-regulated protein, trans-1,2-dihydrobenzene-1,2-diol dehydrogenase, and actin were identified as prominent diisocyanate-conjugated proteins through use of a combination of immunocytochemical and mass spectrometric techniques. Following *in vivo* inhalation of an HDI aerosol, keratin 18 was also identified as the predominant diisocyanate-conjugated protein in human endobronchial biopsy samples, whereas albumin was the predominant diisocyanate-conjugated protein in bronchoalveolar lavage fluid. Keratin was also identified as a predominant diisocyanate-conjugated protein in human skin biopsy samples after epicutaneous exposure to liquid-phase HDI, although the major skin diisocyanate-conjugated protein (56-kD) differed from the predominant lung diisocyanate-conjugated keratin (47-kD). The data from this study identify keratin and other proteins as potential "carriers" for diisocyanates *in vivo*, and suggest that HDI conjugation of these proteins may play a role in the pathogenesis of diisocyanate-induced asthma.

Diisocyanates are a leading cause of occupational asthma in industrialized nations, where they are used in the commercial production of polyurethane-based products. Diisocyanates are uniquely suited for polyurethane production, since their $N=C=O$ moieties react with the OH groups of polyols to form polyurethanes efficiently, with virtually no byproducts. Hexamethylene diisocyanate (HDI), the focus of the present report, is the most commonly used aliphatic diisocyanate (1).

The chemical reactivity of diisocyanate that has been exploited for the commercial production of polyurethane has also hampered *in vitro* and *in vivo* studies of diisocyanate-induced allergy and asthma. Diisocyanates react rapidly with the OH groups of polyols as well as with the OH groups of water, and with functional groups present on all proteins (1). Diisocyanates react with amines, hydroxyls, and sulfhydryls, with the last of these reactions favored under "physiologic conditions" *in vivo* (1, 2). This reactive nature of diisocyanates has obscured studies of the immunopathogenesis of diisocyanate-induced asthma, owing to uncertainty about the antigenic form of the chemicals that trigger allergic reactions.

Most immunologic studies of diisocyanate-induced asthma have so far focused on the hapten-like nature of diisocyanate conjugated to albumin as a carrier protein. These studies have documented the presence of IgE that binds to diisocyanate-albumin adducts in some but not all patients with diisocyanate-induced asthma (3–5). Increased lymphocyte proliferative responses to diisocyanate-albumin adducts have also been documented in some patients with diisocyanate-induced asthma, but the magnitude of this response is significantly smaller than that induced by other allergens, such as dust mite or pollen (6, 7). Few human studies to date have investigated the antigenicity of diisocyanates conjugated to carrier proteins other than albumin, and the importance of the carrier moiety in determining the antigenicity of diisocyanate-protein adducts remains unclear (8–10).

Recently, we reported studies suggesting that HDI-conjugated epithelial cell proteins may be important antigens in diisocyanate-induced asthma. We have shown that: (1) HDI can be detected along the epithelial cell boundary of the airways of exposed human subjects; (2) lung epithelial cell proteins can act as carrier proteins for HDI; and (3) HDI-conjugated human lung epithelial-cell proteins specifically induce proliferation of peripheral blood mononuclear cells (PBMC) obtained from patients with diisocyanate-induced asthma (11, 12). These data suggest that human airway epithelial cell proteins may be important targets for diisocyanate conjugation and entry into the human body, and may play a primary role in the pathogenesis of diisocyanate-induced asthma. Therefore, the objective of the present study was to identify the specific human lung epithelial cell proteins that become conjugated with HDI after exposure *in vitro* and *in vivo*. The study also investigated skin proteins susceptible to HDI conjugation, since skin is also a primary site of occupational exposure to HDI. The implications of the study data with regard to understanding of the immunopathogenesis and diagnosis of diisocyanate-induced asthma are discussed.

METHODS

Reagents

A549 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). HDI, monoclonal anticytokeratin p18 antibody, monoclonal antipancytokeratin (mixture) antibodies, key-hole limpet hemocyanin (KLH), and protease inhibitor cocktail were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), gentamicin, L-glutamine, and nonessential amino acids (NEAA) were obtained from Gibco-BRL (Grand Island, NY). Triton X-100 was obtained from Calbiochem (La Jolla, CA); horseradish peroxidase (HRP)-conjugated antirabbit Ig and antimouse Ig from Pharmingen (San Diego, CA); enhanced chemiluminescence (ECL) Western blotting detection reagents from Amersham (Cleveland, OH); Sepharose-4B from Pharmacia (Piscataway, NJ); Immo-

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bilon-P membranes from Millipore (Bedford, MA); and nitrocellulose beads from BioRad Laboratories (Hercules, CA).

HDI Exposure of A549 Cells

Exposure of A549 human lung epithelial cells to HDI *in vitro* was done as previously described (12). Briefly, A549 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, L-glutamine (2 mM), NEAA (1 mM), and gentamicin (10 µg/ml). Cells were grown to confluence in a 75-cm² tissue culture flask, washed with phosphate-buffered saline (PBS), and exposed to HDI in PBS (containing 1 mM CaCl₂ and 1 mM MgCl₂ to prevent detachment). HDI was serially diluted in acetone before being added to the cells in a volume of 10 ml of PBS with a final acetone concentration of 0.5% (vol/vol) and final HDI concentrations ranging from 19 to 300 µM (0.0003% to 0.005% [vol/vol]). Samples were shaken vigorously and rocked for 20 min at room temperature. Control flasks received vehicle in the same volume. The cells were washed three times with PBS, scraped, and resuspended in 3 ml of PBS or Tris buffer with protease inhibitors.

Crude Fractionation of A549 Cells

Unexposed control or HDI-exposed A549 cells suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100, 1 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 0.1 mM MgCl₂ were sonicated twice with 30-s pulses, followed by a 30-s rest, on ice on a Bronson Ultrasonicator (Bronson Sonicor Co., Danbury, CT). The cells were centrifuged for 30 min at 12,000 × g, and the supernatant and pellet fractions were separated.

Generation and Characterization of HDI Antisera

HDI-specific antisera were raised in rabbits against HDI-KLH adducts as previously described (12). Briefly, HDI-conjugated KLH was emulsified in a 1:1 ratio with complete Freund's adjuvant, and a total of 500 µg of immunogen was given intramuscularly at four different sites to a New Zealand White rabbit (Millbrook Farms, Rehoboth, MA). Following the initial immunization, rabbits were boosted at 3-wk intervals with 250 µg of immunogen in incomplete Freund's adjuvant. Rabbit antisera were generated and processed by the Yale University Immunization Services in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23, as revised). To remove serum antibody reactivity directed at KLH carrier-specific epitopes, the antisera were twice passed over a KLH-coupled Sepharose bead column. A 5-ml column of KLH-coupled Sepharose was prepared, using cyanogen bromide-activated Sepharose 4B according to the manufacturer's directions, with a 5 mg KLH/ml bed volume of beads. Five milliliters of antisera were depleted per column. For dot-blot characterization of the processed antisera, 2 µg of human serum albumin (HSA), HDI bound to HSA (HDI-HSA), A549 cells, HDI-conjugated A549 cells, KLH, and HDI-KLH, generated as previously described (12), were spotted on nitrocellulose strips. The strips were probed with preimmune sera, precolumn (anti-HDI-KLH) sera, the KLH-depleted fraction of antisera, and the KLH-specific fraction of antisera at a 1:1,000 dilution in 5% dry milk, and were developed as subsequently described.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

HDI-exposed and unexposed A549 cells were separated on 7.5 to 12.5% polyacrylamide gradient gels and transferred onto Immobilon-P membranes as described (12). The membranes were probed with rabbit polyclonal anti-HDI antisera (1:2,000) and a secondary HRP-conjugated antirabbit Ig (1:2,000), and were developed with ECL. Fractionated A549 cells were transferred and probed similarly, and some blots were later stripped (according to the manufacturer's protocol) and reprobed with antikeratin antibodies (1:2,000) and a secondary HRP-conjugated antimouse Ig antibody (1:2,000). Protein bands were visualized by Coomassie-R250 staining according to the manufacturer's instructions, excised, electroeluted, and reanalyzed by matrix-assisted laser desorptive ionization mass spectrometry (MALDI-MS) and Western blotting. The Yale Keck Center (New Haven, CT) performed the MALDI-MS.

Human Subjects and HDI Exposure

The part of the study involving human subjects was approved by the Human Investigations Committee of Yale University, and informed written consent was obtained from each subject. Three automobile body shop workers with known prior workplace exposure to HDI and two atopic asthmatic (non-HDI-exposed) subjects were studied. Subject 1 was a 22-yr-old male employed for 6 yr in an automobile body shop. Subject 2 was a 67-yr-old male who had worked in an automobile body shop for the preceding 25 yr. Subject 3 was a 47-yr-old male employed for 21 yr in an automobile body shop, but for the 4 yr preceding the study as an office manager. None of the automobile body shop workers had asthma as based on methacholine testing, peak flow measurements, and questionnaires. Control Subjects 4 and 5 were 26-yr-old and 27-yr-old individuals with atopic asthma (non-HDI-induced), and were skin prick test-positive for mite and cat allergens. Given the risks of HDI sensitization, we did not expose the control subjects with non-HDI-induced asthma to HDI in this study. HDI aerosol exposure of automobile body shop workers was done with a closed-circuit system that delivers HDI biuret aerosol. The closed-circuit apparatus consists of a temperature- and humidity-controlled HDI aerosol generation system and a Teflon-coated Plexiglas mixing cylinder with a face mask delivery system (13). HDI aerosol and vapor levels in the closed-circuit HDI delivery chamber were quantitated with Isochek samplers (ESA Laboratories Inc., Lowell, MA) and an Autotest continuous isocyanate monitor (GMD Systems Inc., Pittsburgh, PA). All three automobile body shop workers received a 2-h exposure to HDI at 20–30 ppb. Thirty minutes after HDI exposure, the subjects underwent bronchoscopy with bronchoalveolar lavage (BAL) and multiple airway biopsies as previously described (11). Control (non-HDI-exposed) subjects underwent identical bronchoscopy. The bronchoalveolar lavage fluid (BALF) was subjected to low-speed (2,500 × g) centrifugation, and soluble proteins were concentrated 30-fold with a 10-kD-cutoff Millipore filter before use. Biopsy samples were snap frozen in liquid N₂ and later homogenized and sonicated as described earlier for A549 cells. Protein concentrations in the patient samples were measured with a kit from BioRad. For BALF and biopsy studies, 20 µg of protein per lane were run on a minigel and subjected to Western blotting as subsequently described.

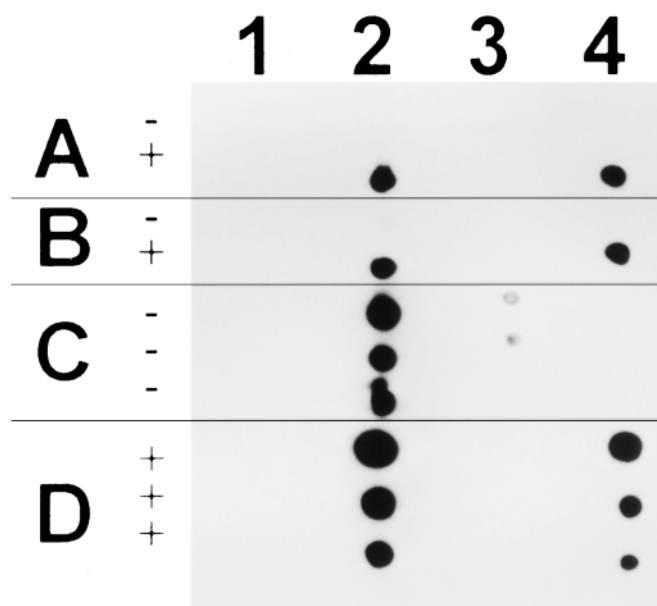


Figure 1. Dot-blot characterization of HDI-specific antisera. Antisera raised against HDI-KLH were dot blotted as described in METHODS against unconjugated (–) and HDI-conjugated (+) proteins including A: albumin; B: epithelial cell proteins; C: titrated KLH; and D: titrated HDI-KLH. Reactivity of preimmune sera (lane 1), HDI-KLH immune serum (lane 2), the anti-KLH fraction of HDI-KLH antisera (lane 3), and the anti-KLH-depleted fraction of HDI-KLH antisera (lane 4) are shown.

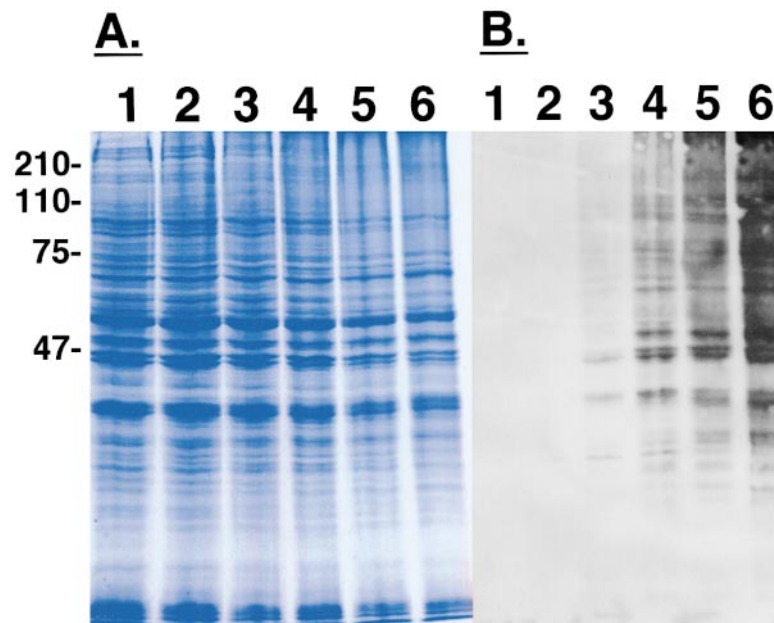


Figure 2. SDS-PAGE and Western blot with anti-HDI antisera of A549 cells exposed to HDI dose titration. (A) A549 cells unexposed (lane 1) or exposed to HDI concentrations of 19 μ M (lane 2), 38 μ M (lane 3), 75 μ M (lane 4), 150 μ M (lane 5), or 300 μ M (lane 6), separated by SDS-PAGE, and stained with Coomassie blue. (B) Parallel Western blot with anti-HDI antisera of the cell proteins shown in A.

Epicutaneous exposure of human Subjects 2 and 3 was done by applying 100 μ l of 0.1% (6 mM) HDI diluted in acetone and olive oil at a ratio of 4:1 to the back, or by applying vehicle alone as a control. Thirty minutes after HDI was applied, 0.5-mm punch biopsies were obtained from unexposed and exposed sites, following local anesthesia with 1% lidocaine. Skin biopsy samples were homogenized and sonicated, and 20 μ g of protein per lane were run on a minigel and subjected to Western blotting as described earlier.

RESULTS

HDI Binding to Human Lung Epithelial Cell Proteins *In Vitro*

We used an immunocytochemical technique to detect human lung epithelial cell proteins bound by HDI after exposure *in vitro*. HDI-specific antibody preparations were raised against HDI-KLH adducts and were characterized by dot-blot assay

before and after preabsorption with KLH columns to remove anti-KLH activity (Figure 1). Western blots of total human lung epithelial cell proteins (A549 cells) done with anti-HDI antisera after a dose-titration exposure to HDI in liquid phase revealed detectable HDI-protein conjugation at exposure concentrations $\geq$ 38 μ M (Figure 2). At relatively low doses (38 to 75 μ M), HDI-protein conjugation did not correlate with protein levels (as detected by Coomassie staining), suggesting that certain proteins are more susceptible to reactivity with diisocyanates than others, including polypeptides of $\leq$ 27 to kD, 36-kD, and 47-kD (Figure 2, lanes 3 and 4). At higher doses (300 μ M), the HDI-protein conjugation pattern more closely reflected relative protein levels, although some proteins still appeared to be disproportionately conjugated with HDI. Parallel sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that HDI exposure also decreased the Coomassie staining of human lung epithelial

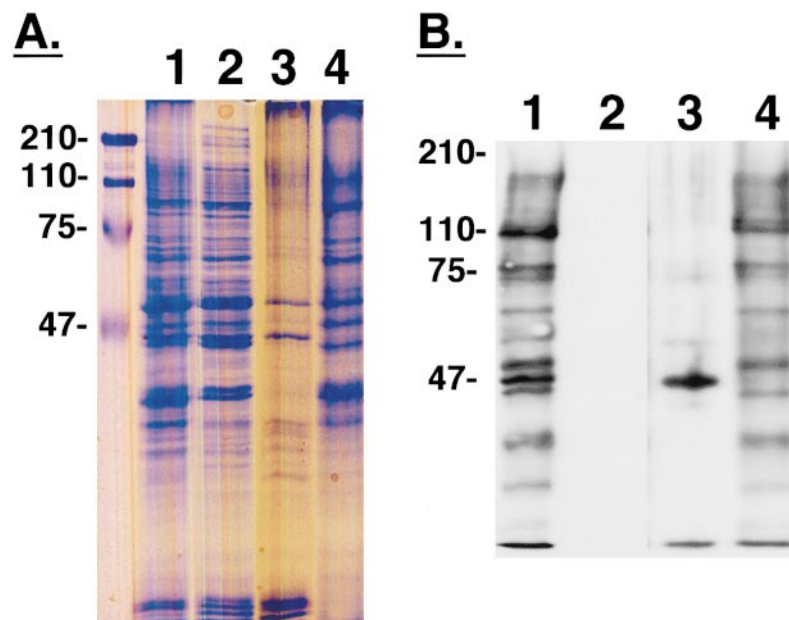


Figure 3. Purification of a 47-kD HDI-conjugated human lung epithelial cell protein. (A) Coomassie blue-stained SDS-PAGE gel of total cell proteins from HDI-exposed (lane 1) and unexposed (lane 2) A549 lung epithelial cells or from the 12,000 $\times$ g pellet (lane 3) and supernatant (lane 4) fractions of HDI-exposed cells. (B) Parallel Western blot, with anti-HDI antisera, of the cell proteins shown in A.

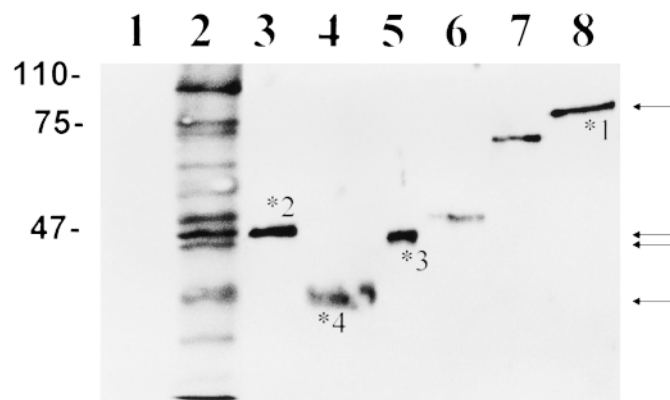


Figure 4. Purification of HDI-conjugated proteins from human lung epithelial cells exposed to HDI *in vitro*. Total proteins from unexposed (lane 1) or HDI-exposed (lane 2) A549 cells, and electroelution purified proteins (lanes 3 to 8) from HDI-exposed A549 cells, were Western blotted with anti-HDI antisera.

cell proteins, which may have been related to HDI interference with Coomassie binding to the proteins.

Identification of Diisocyanate Bound Human Lung Epithelial Cell Proteins

We identified predominant HDI-conjugated human lung epithelial cell proteins by mass spectrometry of proteins from HDI-exposed A549 cells separated by SDS-PAGE. One of the predominant HDI-conjugated proteins was greatly enriched in a $12,000 \times g$ pellet fraction of exposed A549 cells (Figure 3). This 47-kD protein was determined to be keratin 18 through MALDI-MS, and this was further verified by parallel Western blotting with an monoclonal anti-keratin 18-specific antibody (data not shown). Additional HDI-conjugated proteins were identified by first excising and electroeluting, from SDS-PAGE gels, protein bands that corresponded to prominent bands on parallel anti-HDI Western blots. These purified proteins were then simultaneously reanalyzed by Western blotting and MALDI-MS. The 78-kD glucose-regulated protein (GRP78), actin, and trans-1,2-dihydrobenzene-1,2-diol dehydrogenase were identified by this approach (Figure 4). Two of the predominant HDI-conjugated proteins in exposed human airway epithelial cells, of ~ 100 -kD and ≤ 27 -kD, could not be identified by the algorithmic criteria of MALDI-MS.

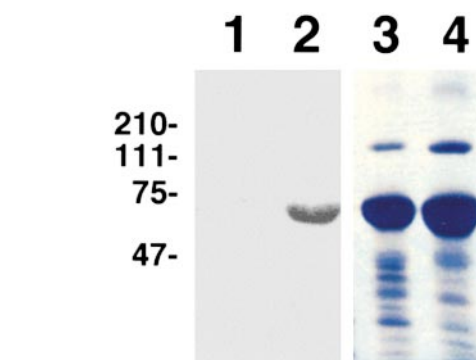
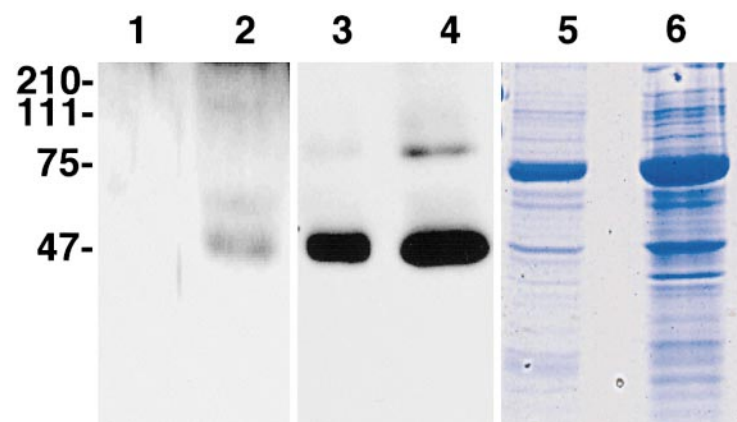


Figure 6. Western blot and SDS-PAGE of BALF from unexposed and HDI aerosol-exposed subjects. BALF from a representative unexposed (lane 1) and an HDI-exposed (lane 2) subject were subjected to Western blotting with anti-HDI antisera. Lanes 3 and 4 show parallel SDS-PAGE gels stained with Coomassie blue.

Detection of HDI-Bound Proteins in Human Tissues Following Exposure *In Vivo*

To determine whether HDI binds to the same proteins *in vivo* as *in vitro*, we probed human airway endobronchial biopsies, BALF, and skin biopsy samples after HDI exposure *in vivo*. Subjects were exposed via airways to 20–30 ppb aerosolized HDI and via skin to 0.1% (vol/vol) (6 mM) HDI, both of which are concentrations of HDI that might be encountered in the workplace (14).

In human airway biopsy samples obtained from subjects exposed to aerosolized HDI, we detected a 47-kD HDI-conjugated protein that comigrated with keratin 18 in parallel Western blots probed with HDI-antisera and a monoclonal anti-keratin 18-specific antibody (Figure 5). A single HDI-conjugated protein was detected in human BALF from subjects exposed to aerosolized HDI (Figure 6), and was probably albumin, on the basis of an electrophoretic mobility corresponding to 68-kD and parallel Coomassie stains. Albumin has previously been shown to be the major diisocyanate-conjugated protein in BALF from guinea pigs and rats exposed *in vivo* to toluene diisocyanate (TDI) in studies using similar immunocytochemical methods to those used in our study, as well as radioisotope tracing methods (15, 16).

We also identified two predominant HDI-conjugated protein bands in human skin biopsies from epicutaneously exposed subjects, one at 56-kD and one > 97 -kD (Figure 7). These HDI-conjugated proteins comigrated with keratin proteins in serial blots that were first probed with anti-HDI anti-

Figure 5. Western blot and SDS-PAGE of airway biopsies from unexposed and HDI aerosol-exposed subjects. Biopsy samples from a representative unexposed (lanes 1, 3, 5) and an HDI-exposed (lanes 2, 4, and 6) subject were subjected to Western blotting with anti-HDI antisera (lanes 1 and 2) or monoclonal anti-keratin 18 antibody (lanes 3 and 4). Lanes 5 and 6 show parallel SDS-PAGE gels stained with Coomassie blue.

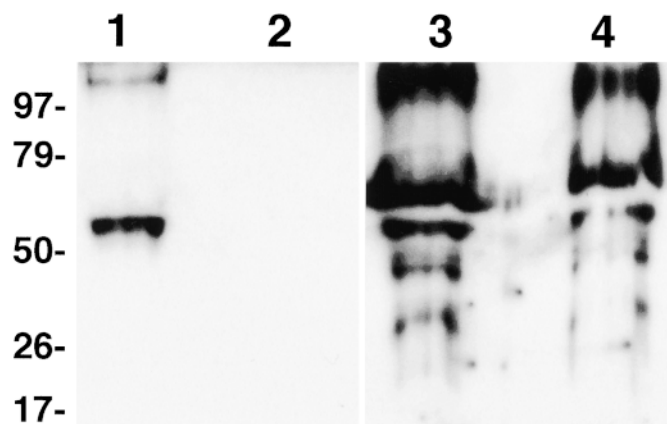


Figure 7. Western blot of unexposed and HDI-exposed human skin biopsies. Unexposed (lanes 1 and 3) and HDI-exposed (lanes 2 and 4) skin punch biopsies from a representative subject were subjected to Western blotting with anti-HDI antisera (lanes 1 and 2) or a panreactive antikeratin antibody cocktail (lanes 3 and 4).

serum and then stripped and reprobed with panreactive anti-keratin antibodies.

The results described here suggest that keratins and albumin were the primary HDI-conjugated proteins in the airways and skin of the HDI-exposed subjects tested. Further studies are needed to determine whether these same proteins are equally susceptible to diisocyanate conjugation in all exposed workers, or whether individual differences in HDI-protein conjugation are related to the development of diisocyanate-induced asthma.

DISCUSSION

Conjugation of diisocyanate to human proteins after occupational exposure is probably an important primary event in the development of diisocyanate-induced allergy and asthma. In the present study, through mass spectrometric and immunocytochemical techniques, we identified human lung and skin proteins that became conjugated to HDI after exposure. HDI-conjugated proteins were detected in human airway epithelial (A549) cells exposed *in vitro* to doses of HDI $\geq 38 \mu\text{M}$, and were also detected in airway and skin biopsies from three subjects exposed to 20–30 ppb aerosolized HDI or 0.1% (6 mM) HDI in the liquid phase, both of which are concentrations of HDI encountered in the workplace (14). Keratin 18 was identified as a predominant HDI-conjugated protein in human airway cells exposed to HDI both *in vivo* and *in vitro*, and is probably the “predominant 47-kD HDI conjugated epithelial cell protein” previously described (12). Additional human airway proteins susceptible to HDI conjugation include the 78-kD glucose-regulated protein, trans-1,2-dihydrobenzene-1,2-diol dehydrogenase, and actin. In BALF from inhalation-challenged subjects, albumin was identified as the major HDI-conjugated protein. In exposed human skin biopsies, we detected a 56-kD HDI-conjugated protein that comigrated with keratin in Western blot analyses.

The newly identified HDI-conjugated human proteins could contribute to the pathogenesis of diisocyanate-induced asthma through several different pathways. One possibility is that the conjugated proteins may serve as carrier proteins that present diisocyanates to the human immune system in a haptenlike manner. Normally intracellular and/or structural proteins (such as keratin or GRP78) could gain access to the immune system following cell death or damage to the epithelial layer that results

in loss of its integrity. Alternatively, normal cellular proteins, if “damaged” by HDI conjugation, could be processed by endogenous pathways, resulting in the presentation of HDI along with molecules of the major histocompatibility system. A second possibility is that diisocyanates directly crosslink cellular proteins in a way that alters their conformation and makes them immunogenic. A third possibility is that HDI conjugation modulates the function of proteins, which may lead to immune activation. Alternatively, HDI metabolites, resulting from interactions with epithelial cell proteins, may be immunomodulatory.

Of the HDI-conjugated proteins newly identified in the present study, the one most interesting with regard to its potential for participation in the immunopathogenesis of diisocyanate-induced asthma may be keratin. The HDI-conjugated keratin that we identified in exposed airway cells, keratin 18, is highly expressed in the lung, where it heteropolymerizes with keratin 8 (17). A likely candidate for the 56-kD HDI-conjugated protein detected in exposed human skin is keratin 10, given its size and predominance in the outermost layers of human skin, the primary site of epicutaneous diisocyanate exposure (18). Both keratin 18 and keratin 10 are type I (acidic) keratins, rich in cysteines and with a highly conserved core region (19). Type I keratins are structurally related to all intermediate filaments and have well-described roles in maintaining cellular integrity and epithelial boundaries (19). Although the potential pathogenic effects of diisocyanate-keratin conjugation are unknown, conjugation of HDI to keratin could affect the function of the latter in maintaining epithelial integrity. It is also possible that keratins act as “carrier” proteins for diisocyanate, or that diisocyanate crosslinking of keratins may cause the formation of “neoepitopes.” The identification of HDI-conjugated keratins after both airway exposure to aerosolized HDI and skin exposure to liquid HDI may help explain the previously reported link between skin sensitization to diisocyanates and the induction of airway hyperresponsiveness to these chemicals in guinea pigs (20). Furthermore, if the antigenicity of diisocyanate depends on the carrier protein, as classical hapten studies have shown for other T-cell responses, then HDI-conjugated keratin may represent a major class of previously unrecognized HDI antigens.

Additional HDI-conjugated proteins were detected in human lung cells exposed to HDI *in vitro* as compared with airway biopsies from subjects exposed to HDI *in vivo*. It remains unclear whether these differences in HDI-protein conjugation after *in vitro* and *in vivo* exposure are related to HDI exposure dosage, the sensitivity of HDI detection by Western blotting, or differences in normal airway epithelial cells as compared with cell lines. Ethical and practical limitations of obtaining larger sample sizes of airway tissue from exposed humans have limited attempts to further investigate these differences. However, preliminary studies in our laboratory, done with animal models, suggest that these additional proteins may also become conjugated to HDI *in vivo*, but that their turnover may occur more rapidly than occurs *in vitro* (unpublished observations).

One of the HDI-conjugated proteins identified after *in vitro* exposure of human airway epithelial cells was glucose-regulated protein (GRP)78, which belongs to a family of stress-inducible GRPs that show molecular and functional homology with heat-shock proteins and bind Ca^{2+} (21). GRPs have been implicated in the development of cytotoxic immunity, and are known to function as molecular “chaperones” in the trafficking of other “damaged” proteins (22, 23). Given their recognized ability of isocyanates to be transferred from protein to protein via “thiol shuttling” (2), we hypothesize that diisocyanates could become covalently linked to GRP78

while GRP78 is trafficking another diisocyanate-damaged protein. Interestingly, GRP78 has previously been shown to be associated with keratin intracellularly (24), and stress proteins homologous to GRP78 have been found to act as molecular chaperones in antigen processing (25). GRP78 could participate in HDI-induced immunopathology by acting as a carrier for diisocyanate, or might be involved in processing diisocyanate epitopes for presentation to T cells.

The 37-kD HDI-conjugated protein in exposed human airway epithelial cells was identified as a product of the dihydrodiol dehydrogenase gene family. Dihydrodiol dehydrogenases, members of the aldo-keto reductase superfamily, are believed to be involved in endogenous steroid metabolism, and exhibit tremendous polymorphism (26, 27). Aldo-keto reductases, such as dihydrodiol dehydrogenases, are ubiquitously expressed, and were first recognized for their role in the metabolism of polycyclic hydrocarbons, which are structurally related to diisocyanates (28). Dihydrodiol dehydrogenases may also effect keratin expression, and have been linked to cellular differentiation and Ca^{2+} fluxes in skin (18).

The 42-kD HDI-conjugated protein in exposed human airway epithelial cells was identified as actin. The conjugation of HDI with actin in exposed cells is notable, given a recent report describing the colocalization of TDI and tubulin in exposed human airway cells (29) and the well-established association of tubulin and actin. Additional studies should determine whether actin serves as a carrier protein for diisocyanate *in vivo*, or whether diisocyanate-actin conjugation could cause cytoskeletal functional impairments that affect the immunogenicity of diisocyanate.

In addition to the HDI-conjugated proteins that we identified in exposed human tissue samples, we identified albumin as the predominant soluble extracellular HDI-conjugated protein in airway lavage fluid from aerosol-exposed subjects. Albumin from BALF has previously been shown to be conjugated with diisocyanate in TDI-exposed animals (15), and serum albumin conjugated with methyldiphenyldiisocyanate has been reported in exposed animals and human workers (16, 30). In revealing HDI-conjugated albumin in BALF of exposed human subjects, our results are consistent with these findings. However, the role of HDI-conjugated albumin in the pathogenesis of diisocyanate-induced asthma remains unclear. Although the kinetics of isocyanate reactivity with albumin in exposed airways are favorable, given that albumin is the predominant protein in airway fluid, the fate of soluble HDI-albumin in airway fluid, and its interaction with the immune system in diisocyanate-induced asthma, remain uncertain.

In summary, we have identified several human proteins that are likely targets for diisocyanate conjugation following exposure to HDI. HDI conjugation to keratins, albumin, actin, a stress protein, and a metabolic enzyme was observed at exposure doses equivalent to or below those that might be encountered in the workplace. The HDI-conjugated proteins that we identified may represent markers of exposure, and may participate in the pathogenesis of diisocyanate-induced asthma by serving as carrier proteins. Alternatively, diisocyanate conjugation might affect the expression or function of the identified proteins, which could contribute to the development of asthma. Further studies are needed to determine whether significant differences in HDI-protein conjugation occur in workers who develop diisocyanate-induced asthma as compared with workers who do not, or whether the pathogenesis of diisocyanate-induced asthma depends on individual differences in the immune response to the HDI-conjugated proteins identified in our study.

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