Development of Immunoassays for Biomonitoring of Hexamethylene Diisocyanate Exposure

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Hexamethylene diisocyanate (HDI) is used widely to manufacture polyurethanes for paints and coatings. It is an irritant and a chemical asthmagen. The U.S. Occupational Safety and Health Administration time-weighted average permissible exposure limit is 5 ppb and the ceiling limit is 20 ppb. We sought to develop a sensitive and specific immuno-bioassay to supplement workplace air monitoring and detect recent HDI exposure. For this, we produced rabbit antiserum to HDI-adducted keyhole limpet hemocyanin (HDI-KLH). The specificity of the antiserum was demonstrated by its reaction with a variety of HDI-conjugated proteins and the absence of reactions with conjugates of other diisocyanates, namely toluene diisocyanate and diphenyl methylene diisocyanate. Four immunoassays were developed and compared for their ability to detect decreasing quantities of HDI-adducted human serum albumin (HSA) containing 2 mol HDI adduct per mol HSA (HDI_2-HSA) as determined by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry. The sensitivities of some of the assays are within the range (0.82–45 nM) of current analytic methods. A Western analysis procedure has a sensitivity of 600 nM HDI adduct on HSA. ELISA inhibition assay, in which microtiter plates are coated with the HDI_2-HSA antigen, has a sensitivity of 300 nM HDI adduct. An immunoblot assay has a sensitivity of 9 nM HDI adduct. The most sensitive bioassay (1.8 nM HDI adduct) is a three-antibody sandwich ELISA in which wells of microtiter plates are coated with the IgG fraction of the anti-HDI-KLH antisera. Compared with analytic methods for HDI biomonitoring, the immunoassays are faster and less costly and accommodate numerous samples simultaneously. The assays have the potential to affect industrial biomonitoring programs significantly. *Key words*: biomonitoring, HDI, immunoassay, occupational asthma. *Environ Health Perspect* 109:1103–1108 (2001). [Online 11 October 2001]
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Isocyanates are highly reactive compounds used to manufacture polyurethanes. They account for the greatest number of reported cases of occupational asthma both in the United States and in other developed countries. Hexamethylene diisocyanate (HDI), one of the most commercially important isocyanates, is used as a polymerizing agent in polyurethane spray paint and coating formulations.

Primary prevention of isocyanate asthma is problematic because industrial exposures are widespread and difficult to characterize, measure, and control. Secondary and tertiary prevention opportunities are limited because no simple way has been found to diagnose isocyanate asthma or identify specific at-risk groups. In addition, there is inadequate knowledge of isocyanate exposure patterns and the factors that both cause and exacerbate the disease.

As a result of its reactivity and recognized toxicity, there is great interest in developing sensitive methods for detection of human exposure to HDI. Determination of HDI in the blood, serum, urine, and other body fluids is difficult because HDI not only is self-reactive buy may also hydrolyze or react readily with hydroxyl, sulfhydryl, or amino groups on proteins.

Exposure to HDI often occurs in small shops or garages where workplace monitoring may be inadequate. In addition, measurement only of airborne concentrations is likely inadequate because animal studies have indicated that exposure through the skin can result in respiratory sensitization (*1*). Biomonitoring presents an attractive way to supplement air monitoring. By integrating the burden from all routes of exposure, biomonitoring can estimate the total internal dose of the agent.

Few biomarkers are available for distinguishing exposure to HDI. Previous methods have used analytic chromatographic procedures to detect hydrolysis products of the isocyanate in urine or blood of workers. Measurement is made of the diamine liberated after acid hydrolysis and extraction of urine and blood samples (*2,3*). Levels of the diamine from methylene diphenyl diisocyanate (MDI) in hydrolyzed plasma ranged from 0.25 to 226 nmol/L (*4*). Toluene diisocyanate (TDI) was also detected as the corresponding diamine in hydrolyzed plasma, with levels of 0.82–45 nmol/L (*5*). Two studies attempted biomonitoring for HDI after exposure of volunteers in test chambers (*2,3*). Both studies failed to detect hexamethylene diamine in hydrolyzed plasma.

We sought to develop a sensitive and specific immunoassay for biomonitoring HDI exposure. Advantages of such an assay are low cost, readily available equipment with low maintenance needs, and the ability to analyze numerous samples simultaneously. We report here the development of immunoassays that have sufficient sensitivity and specificity to detect workplace HDI exposures.

Materials and Methods

Synthesis of HDI–protein antigens. We added 20 μL hexamethylene diisocyanate (Desmodur HD240, cas # 822-06-0, Bayer Corp., Pittsburgh, PA; purity 98%) dropwise to 20 mL of a rapidly stirred 0.5% (w/v) solution of protein in 0.05 M sodium borate–KCl buffer (0.05 M H_3BO_3, 0.05 M KCl, 0.035 M NaOH, pH 9.4) kept at 37°C. After 4 hr, we added 0.5 mL of monoethanolamine to stop the reaction. The solution was filtered, the pH of the supernatant was adjusted to 4.0, and the mixture kept at 4°C overnight. The conjugate was collected by centrifugation, dissolved in 15 mL of 0.1 M $(NH_4)_2CO_3$ and dialyzed against 0.05 M NaCl and then water before lyophilization. Proteins that we reacted with HDI included keyhole limpet hemocyanin (KLH; Sigma Co., St. Louis, MO), human serum albumin (HSA, 97-99% purity; Sigma Co.), and ovalbumin (OVA; Sigma Co.).

We prepared a series of HDI-HSA conjugates by adding increasing amounts of HDI (1–110 μL) to the 0.5% HSA solution. The conjugates were isolated as described above.

Determination of HDI adduction (TNBS analysis). We estimated the extent of HDI coupling to protein from the number of amino groups on the conjugated protein that remained available for reaction with 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma Co.) after HDI treatment. We added 50 mL of freshly prepared 0.03M TNBS to dilutions of 1% solutions of HDI-HSA and HSA in 0.05 M borate-KCl buffer, pH 9.4 (*6*). After 30 min, we measured the absorbance at 420 nm. We calculated the HDI binding as

the percent of protein amino groups that had reacted with HDI as follows:

$$\% \, Substitution = 100 - \frac{[100 \times Absorbance_{420nm} \, of \, HDI \, conjugate]}{[Absorbance_{420nm} \, of \, carrier \, protein]}$$

MALDI-TOF MS analysis. We used a PerSeptive BioSystems Voyager STR matrix-assisted laser desorption time-of-flight mass spectrometer (MALDI-TOF MS) (Framingham, MA) with a high mass charge (*m/z*) detector in the linear mode to collect all of the mass spectra. We dissolved HDI_n-HSA products and the calibrant, bovine serum albumin (BSA) [molecular weight (MW_{ave}) = 66,431], in 50:50 acetonitrile:water and 0.1–0.3% acetic acid. The matrix used for the MS was 10 mg sinapinic acid in l mL 50:50 water:acetonitrile with 0.1% trifluoracetic acid (TFA) or 0.3% acetic acid. We mixed 1:1 sample:matrix directly on the sample plate or in vials with vortexing. One µL was spotted into a well on the 100-well, gold-plated stainless steel sample plate for the analysis. We determined the MS value of n for the HDI_n-HSA adduct by measuring the average increase in mass of the product against a HSA standard. We determined the error in n by a standard deviation calculation from repeat experiments.

Immunization of rabbits. The immunization protocol was similar to that described previously (*7*). Two New Zealand white rabbits were injected intradermally with 1.8 mg HDI-KLH emulsified in 2:3 saline-Freund's complete adjuvant (Gibco BRL, Grand Island, NY). Injections were distributed among 15 sites along both sides of the back. After 21 days, rabbits were boosted subcutaneously with the same dose of HDI-KLH in incomplete adjuvant distributed among 5 sites along the nape of the neck. Blood was drawn 7–10 days following the boost.

Isolation of rabbit IgG. We isolated immunoglobulin G (IgG) from rabbit antisera using a two-step procedure as described by McKinney and Parkinson (*8*). We removed albumin and other non-IgG proteins by precipitation with caprylic acid. We isolated the IgG fraction by adding ammonium sulfate to 45% saturation and then centrifuging at 5,000 *g* for 30 min. The IgG was resuspended in a small volume of PBS and then dialyzed against PBS at 4°C for 16 hr.

Evaluation of antisera using ELISA. We used ELISA to measure the titer of rabbit antibodies to HDI-KLH (*7*). Microtiter plates (Immulon; Dynex Technologies, Inc., Chantilly, VA) were coated by addition of 50 µL of 50 µg/mL HDI-KLH in 0.1 M carbonate buffer, pH 9.6. We added antiserum to wells and made 2-fold dilutions. After incubation at 37°C for 1 hr, we added peroxidase-labeled goat antirabbit IgG (1:3,000, Sigma Co.) and then 2,2-azinobis-[3-ethylbenzo-thiazoline-6-sulfonic acid] (ABTS; Sigma) and H_2O_2. We stopped the enzymatic reaction by adding 50 µL of 0.05% SDS. Absorbance was determined at 410 nm.

ELISA inhibition assay. We performed inhibition assays as described by Jin et al. (*7*). Increasing amounts of test inhibitors dissolved in blotto (PBS containing 0.05% tween 20, 0.5% nonfat dry milk) were added to the wells of antigen-coated microtiter plates before addition of the anti-HDI-KLH antiserum. After 1 hr at 37°C, reagents were added as described above to detect the rabbit IgG. The percent inhibition was calculated as follows:

$$\% \, Substitution = 100 \times \left[\frac{1 - Absorbance_{410nm} \, with \, inhibitor}{Absorbance_{410nm} \, without \, inhibitor} \right]$$

Three-antibody sandwich ELISA (TASE). We coated polyvinyl chloride microtiter plates (Immulon; Dynex Technologies, Inc.) with 0.01 mg/mL (50 µL/well) IgG fraction from rabbit antiserum to the HDI-conjugated KLH diluted in 0.1 M carbonate buffer, pH 9.6. Plates were kept at 37°C for 3 hr and then stored at 4°C. Just before use, wells were blocked by addition of blotto. Samples of HDI-adducted HSA containing dithiothreitol [(DTT) 10 mg/mL; Sigma] were warmed and then added to the wells. After 1 hr at 37°C, we added goat anti-whole human serum (Chemicon International, Inc., Temecula, CA) 1:7,000 in blotto with 0.5% rabbit IgG. We added peroxidase-labeled rabbit antigoat IgG (Sigma) diluted 1:3,000 in PBS-0.05% tween 20 after 1 hr and incubated plates for an additional hr at 37°C. Substrate was added as described above and absorbance determined at 410 nm.

SDS-PAGE of HDI-HSA conjugates. We dissolved lyophilized aliquots of HDI-adducted HSA in sample loading buffer (SLB) composed of 20% of 1.0 M Tris-HCl, pH 6.8; 40% glycerol; 10% of 10% SDS; and 0.8% of 0.5% Coomassie G-250 (Bio-Rad, Hercules, CA). We added 2.46 mg/mL DTT to some of the samples just before use. Samples were heated in a water bath for 5 min at 95°C and then applied to a gradient gel (10–20% Tris-Tricine SDS gel; Bio-Rad). Electrophoresis was performed at constant voltage (100 V, approximately 65 mA).

Western analysis. We placed polyacrylamide gels in transfer buffer (pH 8.3) composed of 192 mM glycine, 25 mM Tris-base, 0.037% SDS, and 20% methanol for 1 hr. We transferred proteins from the gels to nitrocellulose membranes (0.45 µm, Bio-Rad) using a Mini Trans-Blot Cell (Bio-Rad) operated at constant voltage (100 V) for 1.5 hr at 4°C. Membranes were washed 3 times with Tris-buffered saline (TBS, 20mM Tris, 500 mM NaCl, pH 7.5) and blocked overnight at 4°C by immersion in a solution of TBS containing 5% nonfat dry milk (Bio-Rad) with gentle agitation. We added antiserum to HDI-adducted KLH diluted 1:1,000 in TBS containing 0.5% dry nonfat milk and kept the membranes at 10°C for 24 hr. We detected bound antibodies using biotinylated goat antirabbit IgG [Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno-Blot Assay Kit (AAPGAR) Bio-Rad] diluted 1:3,000 in tween-TBS (0.05% tween 20), pH 7.5, and incubation continued for 2 hr at ambient temperature. After washing, streptavidin-biotinylated alkaline phosphatase complex was added, and the membrane was kept at ambient temperature for 2 hr. We added substrate (5-bromo-4-chloro-3-indole phosphate/nitroblue tetrazolium). We stopped the reaction after 2.5 min by rinsing the membrane in distilled water.

Dot blot assay. Samples of HDI conjugate (0.002–4 µg) containing 10 mg/mL DTT were heated in a water bath for 5 min at 95°C and then blotted onto nitrocellulose membranes (0.45 µm; Bio-Rad). We used rabbit IgG isolated from the antiserum raised to HDI-adducted KLH to detect HDI adducts. Spots were visualized as described above for the Western analysis.

Calculation of immunoassay sensitivity. The sensitivity of each immunoassay was calculated as follows:

$$Sensitivity \, (nM) = \begin{aligned} &[minimum \, quantity \, of \, conjugate \, detected \, (\mu g) \\ &\times [\# \, moles \, HDI \, in \, conjugate] \\ &\div [Mol \, weight \, HSA] \times [Vol. \, sample \, (\mu L)] \end{aligned}$$

Results

Specificity of the antiserum to HDI-adducted KLH. Sera from the two rabbits were mixed and evaluated for reactivity with
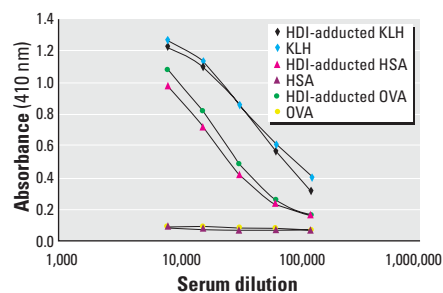
Figure 1. Specificity of the antiserum to HDI-adducted KLH. Each antigen was used to coat a microtiter plate. Bound antibody was detected using 1:3,000 peroxidase-labeled goat antirabbit IgG. The reactivity of the antiserum with all HDI-adducted antigens, but not with HSA or OVA, indicated an anti-HDI hapten titer of 50,000.

HDI-adducted proteins. Using ELISA, we noted reactions with each of the proteins that we had reacted with HDI (Figure 1). Reaction was also detected with KLH, but not with OVA or HSA. The near identity of the titers obtained with HDI-adducted OVA and HDI-adducted HSA indicates that the antiserum has an "anti-HDI" hapten titer of approximately 50,000.

Characterization of HDI-protein conjugates. We anticipated that conjugates formed *in vivo* following occupational exposure would contain a small number of adducts. Accordingly, we sought to prepare a conjugate containing only one or two HDI adducts that could be used to develop the immunoassays. We synthesized conjugated proteins under conditions of physiologic pH and temperature, employing molar ratios of HDI:HSA that ranged from 0.8 to 91.3.

We analyzed the extent of adduction of the conjugate series by three methods. A comparison of the methods is presented in Table 1. Using the TNBS method, we found that adduction was a linear function of the initial HDI concentration. However, some of the conjugates that tested negative in the TNBS assay gave a positive response with the rabbit anti-HDI adduct antiserum (conjugates B and C, Table 1). Because the TNBS reagent assesses primarily chemical adduction with amino groups (*9*), we performed additional analyses using mass spectrometry. We synthesized conjugate A using a molar ratio of HDI:HSA of 45.6:1. MALDI-TOF-MS detected an average of 20.6 mol HDI adduct per mol HSA, whereas TNBS indicated 12 mol adduct per mol protein (Table 1). This difference may

be caused by the reaction of HDI with nucleophilic groups other than amines. The TNBS method could not detect adduction on either conjugate B or C. In the immunologically based Western blot assay, conjugate B gave a moderately strong response and conjugate C showed a weak response.

The MALDI mass spectra in Figure 2 show typical data collected for the HSA control in Figure 2A and for two different conjugates in Figures 2B and 2C. Figures 2B and 2C illustrate conjugates that tested negative with TNBS but positive in the Western blot immunoassay (Table 1). The standard deviation in the MW determination of the calibrant, BSA ($MW_{ave} = 66,431$ amu), from 11 runs was ± 44 amu ($\pm 2\sigma$). Although BSA is not recommended as a calibrant for MALDI-TOF-MS because it is typically not pure and can produce a systematic error, the determination of the number of HDI molecules adducted was calculated by mass difference. The average MW_{ave} of the HSA control determined from six runs by MALDI-TOF-MS was $66,653 \pm 126$ amu. One mass spectrum of the HSA control is shown in Figure 2A with m/z 66,662 for the $[M+H]^+$ ion. This MW is approximately 200 amu higher than expected. We suspect that the HSA may be complexed with fatty acids or modified by other plasma molecules. One of six analyses of conjugate B is shown in Figure 2B labeled with m/z 66,985 for the $[M+H]^+$ ion. The six measurements produced a MW_{ave} of $66,980 \pm 70$ amu for conjugate B, indicating an average of two HDI adducts per molecule of HSA. Figure 2C shows one of six trials used to measure the MW of conjugate C found here with m/z 66,670 for the $[M+H]^+$ ion.

The six measurements of conjugate C produced a MW of $66,664 \pm 70$ amu, indicating an average of 0.1 HDI adduct per molecule of HSA. The error in this latter set of experiments is greater than the determined number of HDI adduct molecules.

Reactivity of the antiserum to HDI-adducted KLH. We used the Western blot procedure to evaluate the reactivity of the antiserum with diverse chemical haptens. HSA was reacted with HDI, with other diisocyanates such as TDI and MDI, and with the industrial chemicals formaldehyde and cyanuric chloride. Each of these HSA-conjugates was evaluated with the TNBS reagent and found to contain a minimum of 17 haptenic moieties per molecule HSA. Each conjugate was subjected to gel electrophoresis, transferred to a nitrocellulose membrane, and probed with the antiserum to determine antibody cross-reactivity. Of the antigens tested, only HDI_2-HSA reacted with the antiserum (Figure 3, lane 6). Two bands were detected, a broad band at 69 kDa and a narrower band at 128 kDa. Both bands were identified as HSA by amino acid sequencing.

The lack of reaction of the antiserum with HSA (lane 1) and with other chemically adducted conjugates (lanes 2–5), and the positive response with HDI_2-HSA (lane 6) highlights not only the specificity of the antiserum for HDI hapten, but also its sensitivity by its ability to react with the hapten when present to the extent of 0.2% of the mass of the conjugate.

Immunoassays for HDI adduct. We developed four immunoassays for biomonitoring HDI-adducted proteins present in fluids. We tested each to determine its sensitivity in detecting HDI adducts on the HDI_2-HSA conjugate. An immunoblot method employed nitrocellulose, to which was applied HDI_2-HSA (heated in the presence of DTT). We visualized adducts using 1:1,000 rabbit anti-HDI–conjugated KLH antiserum, followed by goat antirabbit IgG. As indicated in Figure 4, the antiserum detected from 0.031 to 4.0 μg adducted protein. At concentrations < 0.031 μg, staining

Table 1. Characterization of HSA adduction by HDI using spectroscopic and immunologic procedures.

Conjugate	Molar ratio of reactants HDI/HSA	Number of HDI adducts bound to HSA as determined by		Western blot immunoassay
		MALDI-TOF-MS	TNBS	
A	45.6	20.6 ± 0.9^a	12^a	$++++^b$
B	2.5	2.0 ± 0.9	ND	$+++$
C	0.8	0.1 ± 0.9	ND	$+$

Abbreviations: ND, not detected; TNBS, trinitrobenzenesulfonic acid.
[a]Only portion soluble at pH 4 was analyzed. [b]Number of plus signs (+) denotes relative stain intensity, scale + to ++++.
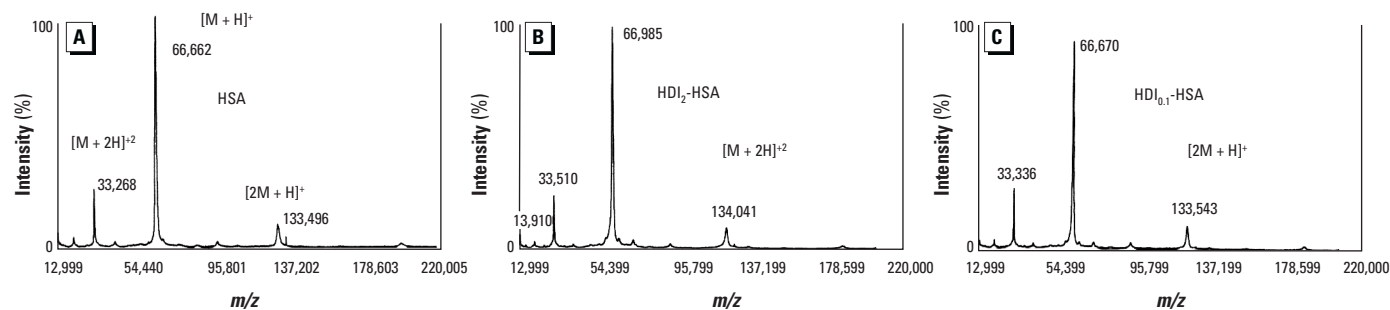

Figure 2. MALDI-TOF mass spectra. Typical mass spectrum of (*A*) human serum albumin control, (*B*) HDI_2-HSA product, and (*C*) $HDI_{0.1}$-HSA product. Note also the assignments of the doubly charged ions $[M + 2H]^{+2}$ and proton-bound dimer ions $[2M + H]^+$ in the spectra.

was not distinguishable from that obtained with HSA. Using the equation provided in "Methods," the sensitivity of this method for detecting HDI adducted to protein is $(0.03 \times 2)/(66{,}500 \times 100) = 9.3$ nM.

We evaluated the Western blot method for its sensitivity in detecting the adduct. We applied quantities of HDI_2-HSA conjugate (0.2–10 µg) to gradient gels. Two bands were present in the HSA starting material (Figure 5A), and we detected HDI adducts on both bands (Figure 5B). The antiserum routinely detected 0.2 µg of HDI-adducted HSA (lane 12), yielding a sensitivity of 600 nM HDI hapten.

We developed an ELISA inhibition method in which HDI_2-HSA (heated under reducing conditions) was used to coat the microtiter plates. Addition of increasing amounts of this conjugate to the wells just before addition of the antiserum yielded a standard inhibition curve (Figure 6). The smallest quantity of inhibitor that could be detected was 0.25 µg (7.6 pmol HDI hapten). The method can detect 300 nM HDI conjugate.

The final immunoassay developed was a three-antibody sandwich ELISA (TASE). In this procedure we used IgG (0.01 mg/mL) purified from the rabbit anti-HDI conjugated KLH antiserum to coat the plates. We added HDI conjugate to the IgG-coated wells. We detected bound HDI hapten using goat antihuman serum followed by peroxidase-labeled antigoat IgG. Detection of HDI_2-HSA is shown in Figure 7. No binding was obtained with HSA. This assay has a
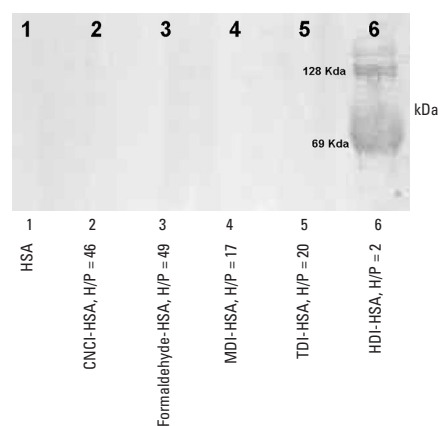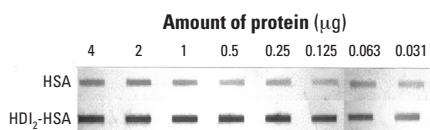
detection limit of 0.003 µg of HDI-adducted protein, yielding a sensitivity of 1.8 nM HDI hapten.

Discussion

1,6-Hexamethylene diisocyanate, also referred to as 1,6-diisocyanatohexane, is a widely used industrial chemical. Its use, coupled with its recognized acute toxicity and chronic consequences, requires careful monitoring of workplace environments. Rapid identification of exposures is essential to prevent adverse health effects. For isocyanates, monitoring the workplace may not be adequate because many exposures are intermittent and occur in small shops or garages where routine air monitors are not installed. Because biomonitoring can integrate exposures that result from diverse routes, such as dermal and inhalation, it presents an attractive supplement to detect workplace exposures.
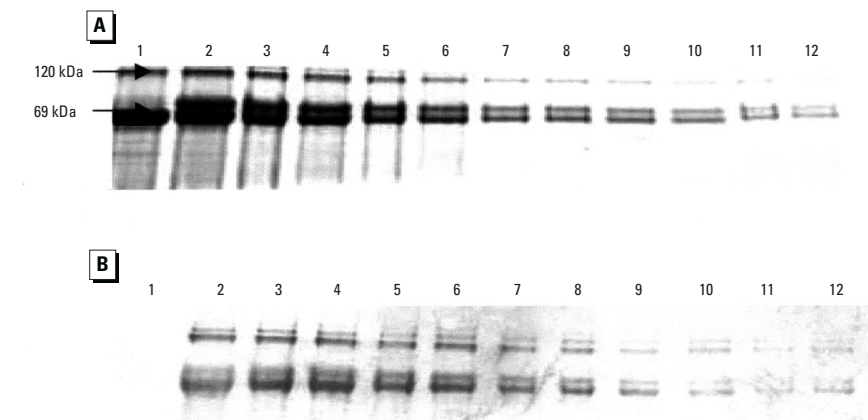
Reliable data regarding human exposure to diisocyanates are lacking. Monitoring airborne TDI concentration has not prevented adverse health effects in exposed workers (10). Biomonitoring may be a valuable supplement. For chemicals such as TDI, which function as haptens, measurement of the immune response, such as the amount of circulating antibody reactive with TDI-conjugated HSA, has been proposed as a means to biomonitor exposure (11). Animal studies have found a direct dependence of the immunologic response on the airborne isocyanate concentration (12). This approach has limitations, however, in that antibodies have not been detected in all exposed individuals (13), and the effect on antibody production of repeated exposures after variable periods of time remains to be clarified. Detection of bound diisocyanate, as we describe here, offers advantages when compared with biomonitoring for the

immunologic response resulting from diisocyanate exposure, because the former measures only recent exposure (within the past several weeks) whereas the immune response may represent exposure occurring over previous months (11).

Of foremost importance in designing a procedure for biomonitoring is adequate sensitivity of the assay. Immunoassays are known to have high sensitivity with antigen detection limits typically in the femtomole range (14). They have been used to study the mutagenic and carcinogenic potential of diverse electrophilic chemicals and their metabolites by assessing DNA adducts (15). Protein adducts, most frequently employing hemoglobin and serum albumin, have been used to gauge exposure to benzene, aromatic amines, and other xenobiotics (16).

Chromatographic methods are currently in use for biomonitoring diisocyanates. TDI and MDI have been detected as the corresponding diamines in acid hydrolyzed urine and blood specimens (3,5). A relationship has been shown between airborne isocyanate levels and concentrations of diamines in hydrolyzed urine from TDI-exposed workers. Using HPLC and GC-MS, detection limits in the biologic matrix of 10 nM have been reported (17).

We report development of four types of immunoassay for detecting HDI adduct. All derive their specificity from the same polyclonal antiserum. By design, the isocyanate hapten is quantified irrespective of the biomolecule to which it is adducted. This is important because the in vivo bioreactant(s) is unknown. In vitro studies have shown that the electrophilic N=C=O moiety reacts rapidly with a variety of nucleophilic groups on amino acids such as terminal amino groups and ε-amino of lysine, thiol in cysteine, phenolic hydroxyl group in tyrosine,
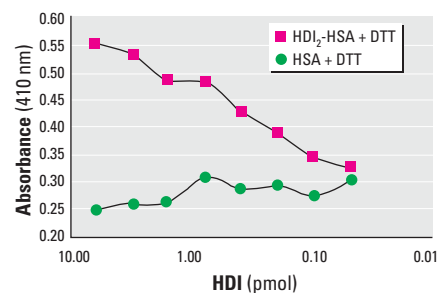

Figure 3. Western blot analysis of antibody cross-reactivity. H/P, hapten/protein ratio. Ten micrograms of protein was loaded into each lane.

1: HSA
2: CNCl-HSA, H/P = 46
3: Formaldehyde-HSA, H/P = 49
4: MDI-HSA, H/P = 17
5: TDI-HSA, H/P = 20
6: HDI-HSA, H/P = 2

Amount of protein (µg)

	4	2	1	0.5	0.25	0.125	0.063	0.031
HSA								
HDI_2-HSA								

Figure 4. Immunoblot assay for detection of HDI hapten.

Figure 5. Western blot assay for detection of HDI adducts. (*A*) Coomassie Blue stain of protein bands. Both the 69 kDa and the 128 kDa bands were identified as HSA. (*B*) Detection of HDI adducts. Protein added to lane 1: 10 mg HSA; to lanes 2–12 HDI-adducted HSA in the following amounts: 10 µg (lane 2), 7 µg (lane 3), 5 mg (lane 4), 3 µg (lane 5), 2 µg (lane 6), 1.0 mg (lane 7), 0.9 µg (lane 8), 0.8 µg (lane 9), 0.5 µg (lane 10), 0.3 µg (lane 11), and 0.2 µg (lane 12).

and the imidazole group in histidine (*18,19*). At pH 7.4 in 50% dioxane, the HDI adduct with N-acetyl cysteine was unstable. Under physiologic conditions, the most likely HDI adducts with proteins are N-terminal amino acids and lysine residues (*18*).

The specificity of the rabbit antiserum was of prime importance in developing the bioassays. We examined its cross-reactivity by testing antibody recognition of adducts formed from other diisocyanates (TDI and MDI), as well as adducts of electrophiles such as formaldehyde and cyanuric chloride. The lack of antibody reaction with any of these hapten-HSA conjugates indicates that antibody specificity is not directed solely to the urea group. The specificity observed in

Figure 6. ELISA inhibition. Values indicate mean ± SEM.

Figure 7. Three antibody sandwich ELISA. Replicate values differed by < 5%.

the current study, together with previous findings (*20*), indicates that hexyl moieties comprise a portion of the epitope. The true epitopes in diisocyanate-conjugated proteins remain unknown, and several different epitopes may exist. This issue is the subject of ongoing studies.

Two of the immunoassays (ELISA inhibition and TASE) detect HDI hapten bound to small molecules (acid soluble amino acids or peptides) as well as to proteins. Recent studies from our laboratory and elsewhere have indicated a favorable, rapid, and reversible reaction of TDI with the sulfhydryl moiety of glutathione. We detected rapid transcarbamoylation upon addition of a sulfhydryl-containing protein to the bis(*S*-glutathionyl) adducts of TDI and HDI (*21,22*). Such adducts, if present in biologic fluids, would be detected by the ELISA inhibition assay and TASE described here.

Workers exposed to airborne TDI had TDI in plasma covalently bound with albumin (*23*). For this reason, we used the albumin conjugate of HDI to develop the bioassays. To parallel the environmental situation further, we synthesized a conjugate that contained a minimum number of HDI adducts. HDI$_2$-HSA was used to develop each of the assays.

We found it necessary to pretreat the HDI conjugates with dithiothreitol before their use. In the immunoblot procedure, the rabbit antiserum to HDI-adducted KLH did not detect conjugates with fewer than four HDI haptenic moieties unless the conjugates were heated under reducing conditions (data not shown). The same effect was reported by Jin et al.(*7*). The reduction of disulfide bonds by DTT likely provides greater accessibility of the antibodies to HDI-adducted regions of the protein.

We compared the four immunoassays for sensitivity and other attributes important

in biomonitoring procedures. As indicated in Table 2, the assays differ in sensitivity by approximately two orders of magnitude. As currently performed, ELISA inhibition and TASE have adequate sensitivity to detect workplace isocyanate concentrations. Moreover, the immobilized immunoglobulins in the latter assay can be an affinity surface to concentrate HDI-adducted molecules from biologic fluids, thereby significantly increasing the sensitivity of the procedure.

The assays described here possess other advantages when compared with current chromatographic methods. The chromatographic determinations rely on acid hydrolysis of biologic fluids to release the diamine. Strong acid is needed, and the amount of amine detected depends on the hydrolysis conditions used. By contrast, the immunoassays require only mild heating of the samples under denaturing conditions. The treated plasma is added directly to the microtiter wells (or nitrocellulose membrane) without need for acid hydrolysis, extraction, or derivatization of product.

Because none of the four immunoassays has been optimized, modification of various parameters may further enhance the sensitivity of each of them. Future work in our laboratory will directly compare findings from the analytic chromatographic methods with results from our immunobioassays to assess the applicability of these immunoassays. Thereafter, protocols applicable for sampling, collecting, and testing in the occupational and environmental setting will be developed.

In summary, we have developed four immunoassays for use in biomonitoring of HDI exposure. The sensitivity of the assays range from 1.8 to 600 nM HDI adduct. By comparison with current analytic methods of biomonitoring for this diisocyanate, the immunoassays are faster, easier, and less costly and require less technical expertise. Most important, they can be employed for routine screening for total HDI exposure and are applicable to industrial surveillance programs to prevent adverse biologic consequences.

Table 2. Comparison of the immunoassays for detection of HDI adducts.

Immunoassay	Sensitivity (nM HDI adduct)	Advantages	Disadvantages
Western blot	600	Indicates the number of carrier proteins adducted and their molecular sizes.	Time consuming.
ELISA Inhibition	300	Detects HDI moieties irrespective of the carrier protein.	Time consuming. Does not indicate nature or number of carrier molecules.
Immunodot	9	Rapid, technically simple procedure for screening a large number of samples.	Does not indicate the nature or number of carrier molecules.
TASE	1.8	Readily quantifiable, highly sensitive.	Suitable for screening a large number of samples. Does not indicate the nature or number of carrier molecules.

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