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Research paper

Production, characterization and utility of a panel of monoclonal antibodies for the detection of toluene diisocyanate haptenated proteins

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ABSTRACT

Diisocyanates (dNCOs) are highly reactive low molecular weight chemicals used in the manufacture of polyurethane products and are the most commonly reported cause of occupational asthma. Mechanistic disease studies and development of biomonitoring and research tools, such as monoclonal antibodies (mAbs) have been hampered by dNCOs' ability to self-polymerize and to cross-link biomolecules. Toluene diisocyanate (TDI)-specific monoclonal antibodies (mAbs), with potential use in immunoassays for exposure and biomarker assessments, were produced and reactivities characterized against mono- and diisocyanate and dithioisocyanate protein conjugates. In general, TDI reactive mAbs displayed stronger recognition of isocyanate haptenated proteins when the NCO was in the ortho position relative to the tolyl group, and were capable of discriminating between isocyanate and isothiocyanate conjugates and between aromatic and aliphatic dNCOs. Preliminary studies using TDI vapor exposed cells suggest potential utility of these mAbs for both research and biomonitoring.

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1. Introduction

Diisocyanates (dNCOs) are commonly used in paints, glues/binders and in the production of polyurethanes. The most common monomeric dNCOs are toluene diisocyanate (TDI), methylene diphenyldiisocyanate (MDI) and hexamethylene diisocyanate (HDI). Workers are exposed to these as well as to polymeric forms of dNCOs. Diisocyanate exposure is the most commonly reported cause of occupational asthma with the prevalence of dNCO-induced asthma estimated at 5–15% in workers (Redlich and Karol, 2002). Due to their widespread use in commercial products available to the general public, actual rates of dNCO-induced diseases may be under-reported. Health aspects of dNCO exposure have been subjected to intensive research, in terms of both human and animal toxicological studies. Dose-dependent responses to higher levels of dNCOs include respiratory, dermal and mucous membrane irritation. Hypersensitivity reactions to dNCOs include allergic rhinitis (Johnson et al., 2007) , asthma (Karol, 1986), hypersensitivity pneumonitis and allergic contact dermatitis (Hur et al., 2008).

Conjugation (haptenation) of diisocyanate to human proteins after exposure is commonly accepted as an important primary event in the development of diisocyanate-induced allergic sensitization and asthma. The major dNCO adducts found in the blood are hemoglobin and human serum albumin (HSA) (Mraz and Bouskova, 1999; Wisnewski et al., 2000; Wisnewski and Redlich, 2001; Johannesson et al., 2004). TDI-conjugated lung proteins in a murine study were co-localized by immunochemical staining with tubulin and actin, which suggests that these proteins may also be targets (Lange et al., 1999). Other skin and lung proteins and peptides like keratin, glutathione and actin have been reported as HDI binding targets (Wisnewski et al., 2005, 2000). The immunogenic protein form(s) that lead to

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sensitization and asthma in the occupational environment cannot be inferred from these studies. It can be concluded from these reports that TDI binding *in vivo* demonstrates selectivity with respect to target proteins; The ultimate antigenic protein (s), and all forms of chemical linkages are, however, not yet known (Ott et al., 2007).

Biological monitoring of exposure to dNCOs involves either the measurement of dNCO-specific antibody in sera or indirect measures of dNCO-conjugated biomolecules in blood or urine. The most commonly used biomonitoring assays estimate total TDI exposure by converting urinary TDI-bound species to toluene diamine (TDA) by strong acid or base hydrolysis. A variety of analytical methods (e.g. chromatography) have been used to determine the amount of TDA generated by laboratory hydrolysis (Chappelle et al., 2002; Sennbro et al., 2004). The detection of TDA in urine samples does not reflect the level of free TDA in the body; rather it estimates the combination of conjugated TDI derivatives and free-TDA (Tinnerberg et al., 1997; Dalene et al., 1994). As a result this method cannot distinguish between TDI and TDA exposure. Sabbioni et al. (Sabbioni et al., 2001) reported a novel dNCO biomarker assay employing mild base hydrolysis of hemoglobin from MDI exposed rats to yield the hydantoin from the MDI conjugated to the N-terminal valine. They also recently produced a more sensitive measure of dNCO exposure by measuring dNCO conjugated lysine in sera after pronase digestion (Sabbioni et al., 2010) and compared sensitivity of the urinary methylene dianiline, hemoglobin hydantoin, and lysine conjugate methods in a population of MDI exposed workers. Methylene dianiline, hemoglobin and lysine conjugates were found in 95.7%, 27% and 64% of the workers, respectively. There is a need for dNCO biomonitoring methods with sufficient sensitivity and specificity to monitor a worker's exposure/body burden. Better biomonitoring methods could provide clues regarding the haptenation events, important for early intervention and prevention of allergic disease.

Multiple hurdles must be addressed for production of relevant dNCO-specific monoclonal antibodies, many of which are the same as for detection of dNCO-specific antibody from the sera of exposed workers as detailed by Ott (Ott et al., 2007). In the present study we report on the production and characterization of TDI specific IgG monoclonal antibodies that specifically recognize 2,4 and/or 2,6 TDI-conjugated biomolecules. These mAbs differentiate between different dNCOs, including MDI, and their reactivities are not dependent on specific conjugated proteins.

2. Materials and methods

2.1. Conjugation of diisocyanates, monoisocyanates (NCOs) and diisothiocyanates (dNCSs) to proteins

All reagents were obtained from Sigma Aldrich Co, St Louis , MO and used without further purification. Conjugations of dNCOs, NCOs and dNCSs to target proteins were carried out as previously described (Ruwona et al., 2010). For immunizations, 2,4 and 2,6 TDI were conjugated to Keyhole Limpet Hemocyanin, KLH, (from *Megathura crenulata*). The conjugates were filtered through 0.45 μm syringe filters (Millipore, Billerica, MA, USA) and stored in aliquots at −20 °C until further use. NCOs, dNCSs and other dNCOs were conjugated only to HSA for mAb binding characterization purposes.

2.2. Conjugation analysis

The dNCOs, NCOs and dNCSs are electrophilic species with the ability to react covalently to multiple nucleophilic sites on a single protein. Each conjugate was assessed for the extent of conjugation by 2 different methods: loss of 2,4,6-trinitrobenzene sulphonic acid, (TNBS, Sigma-Aldrich Co., St. Louis MO) reactivity toward primary amines (Sashidhar et al., 1994) and the shift in protein mass by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS).

2.3. Immunization of mice

Mice were housed under controlled environmental conditions in HEPA-filtered ventilated polycarbonate cages on autoclaved hardwood Beta-chip bedding and were provided food (irradiated Teklab 3517 rodent chow, Harlan, Madison, WI) and autoclaved tap water, *ad libitum*. Sentinel mice were free of viral pathogens, parasites, mycoplasmas, and Helicobacter. The NIOSH facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All research protocols were approved by the NIOSH animal care and use committee. Five female BALB/c mice (10–14-week old) were immunized with a 50:50 (v/v) emulsion of 2,4 or 2,6 TDI-KLH and TiterMax® using 50 μg 2,4 or 2,6 TDI-KLH per mouse at biweekly intervals. KLH was used as the carrier to immunize the mice because of its large molecular weight (polymer, molecular weight over 5 million Da), its high immunogenicity (absence of mammalian homologs) and large number of possible conjugation sites. Tail vein blood samples were taken in 2-week intervals starting 1 week before immunization (prebleed) in order to monitor the development of immune responses.

2.4. Evaluation of TDI-antisera by ELISA

Blood was collected from the tail vein before dosing and every other week between booster immunizations to assess the anti-TDI titers against 2,4 and 2,6 TDI-HSA by ELISA. Screening of sera was conducted at dilutions from 1/200 to 1/12,800 and 100 μL/well for all reagents. dNCO-protein antigen (0.5 μg well) in carbonate coating buffer (60 mM sodium carbonate, 140 mM sodium bicarbonate, pH 9,6) was added to the ELISA plate and incubated at 4 °C overnight. After 24 h. the plates were washed three times with phosphate buffer containing 0.02% Tween-20 (PBST). After washing, 200 μL of 5% nonfat dry milk in PBST buffer (PBSTM) was added to each well and incubated at room temperature for 1 h and then washed three times with PBST. Sera from TDI-KLH treated mice were diluted in PBSTM buffer, added at 100 μL per well and incubated at room temperature for 1 h. After washing, 100 μL of Biotin-SP-conjugated affinity-purified goat anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA) was added at a dilution of 1/5000 in PBSTM and incubated for 1 h at 37 °C. Bound biotin was then detected by adding a 1/5000 dilution of alkaline phosphatase-conjugated streptavidin (Jackson Immuno Research Laboratories, Inc., West Grove, PA) for 1 h at 37 °C in PBSTM. ELISA wells were developed by addition of 100 μL/well of p-nitrophenyl phosphate-containing buffer (2 mg/mL in 1 M diethanolamine, 0.001 M $MgCl_2$ in distilled water, pH 9.8) at RT and the optical density (OD) was determined spectrophotometrically at 405 nm after

a 30 min using an UltraMicroplate Reader, Model ELx800 (BIO-TEK Instruments, Inc., Winooski, VT).

2.5. Monoclonal antibody production

Mice were euthanized by CO_2 asphyxiation three days after the final booster immunization. Spleens were removed aseptically and lymphocytes harvested after lysing red blood cells by osmotic shock. Hybridomas were produced using standard polyethylene glycol-based cell fusion techniques (Harlow and Lane, 1988) using the SP2/0-AG14 myeloma (ATCC# CRL-1581). SP2/0-AG14 myeloma cells were fused with spleenocytes at a ratio of 1:10 myloma:spleen cells using 1000 μL of polyethylene glycol (molecular weight 1500 Da) as the fusogen.

Hybridoma cultures were maintained in Dulbecco's Modified Eagle Medium (Life Technologies, Rockville, MD), supplemented with 1 mM pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin and 0.292 mg/mL l-glutamine, 100 μM sodium hypoxanthine, 16 μM thymidine and 10% fetal calf serum (HyClone, Logan, UT) and 100 units/mL IL-6 (Boehringer Mannheim, Germany). The number of cell clones per well was determined after 5–6 days of cell culture. Culture supernatants from wells with cell growth were screened by ELISA and the cells from positive wells were cloned twice by limiting dilution. Cloned hybridomas were transferred sequentially to 48-well Nunc tissue culture plates, 24-well Nunc tissue culture plates and finally to 50 mL tissue culture flasks to provide culture supernatant for further testing. Aliquots of stable hybridomas were frozen in a mixture of 10% (v/v) dimethylsulfoxide and 90% fetal calf serum before being transferred and stored in liquid nitrogen.

2.6. ELISA format for hybridoma screening

Hybridoma screening tests were carried out using an alkaline phosphatase-mediated indirect ELISA (Schmechel et al., 2006). In brief, MaxiSorp™ ELISA plate wells (Nalge Nunc International, Naperville, IL) were coated with 4 μg/mL of 2,4 or 2,6 TDI-HSA overnight at RT and the plates were kept in a plastic box containing moist filter paper. Following overnight incubation, plates were processed as described above for the evaluation of TDI antisera ELISA. Wells were considered to be positive when optical density (OD) values were 2.5× greater than the OD values of control wells that were processed with fresh tissue culture medium instead of mAb culture supernatants.

2.7. Determination of antibody isotype and antibody quantification

Antibodies were isotyped using a mouse monoclonal isotyping reagent kit (Jackson Immuno Research Laboratories, Inc., West Grove, PA) according to the manufacturers' instructions. In brief, plates were coated with antigen, blocked, and washed as described for the screening ELISA. To determine the isotype, wells with bound mAbs were incubated with 100 μL of Biotin-SP-conjugated goat anti-mouse isotype-specific secondary antibodies (IgG_1, IgG_{2a}, IgG_{2b}, or IgG_3). Monoclonal antibodies were quantified using isotype specific ELISA kits (Jackson Immuno Research Laboratories, Inc., West Grove, PA) according to the manufacturers' instructions. Amount of antibody in supernatant fluid was calculated using a standard curve generated with isotype specific antibodies of known concentrations. The standards and supernatant fluid were assayed in parallel.

2.8. ELISA format for mAb binding specificity studies

Reactivity was assessed by indirect ELISA using an alkaline phosphatase-conjugated secondary antibody. PolySorp™ ELISA plate wells (Nalge Nunc International, Naperville, IL) were coated with 4 μg/mL of test antigen (see Table 1) overnight at room temperature (RT) and plates were processed as described for anti-TDI ELISA. The results represent the mean OD of 4 replicates which were corrected by subtracting the average OD of quadruplicate control wells. Assay background controls were processed in parallel but contained plain culture medium alone instead of mAb supernatant. ODs>2.5 X background HSA wells were considered to be positive (see Table 2).

2.9. ELISA for affinity constant (K_a) determination

Antibody affinity constants were determined according to Beatty et al. (Beatty et al., 1987) with modifications. ELISAs were carried out to determine the antibody and antigen concentration that would result in saturation kinetics. The antigens were used at 2.5 μg/mL, 1.25 μg/mL, 0.625 μg/mL and 0.312 μg/mL and antibody concentration ranged from 1.127×10^{-9} to 7.48×10^{-10} M^{-1} and the ELISA plates were processed as previously described. Curves were generated in SigmaPlot® using a four parameter logistic model (Hill-Slope model) and used calculate OD-50.

Table 1
Characterization of protein adducts to isocyanates using spectroscopic procedures.

Protein and Hapten-Protein	Primary Amine TNBS Assay		MALDI-TOF-MS[1]
	% substitution	NCO/molecule	NCO/molecule
HSA	0	0	0
2,4 TDI-HSA	87	10	23
2,6 TDI-HSA	85	10	43
HDI-HSA	77	8	26
MDI-HSA	66	7	25
PI-HSA	14	2	3
PTI-HSA	21	2	–
OTI-HSA	40	4	5
2,4 TITC-HSA	39	4	4
2,6 TITC-HSA	37	4	2
2,3 DMPI-HSA	83	9	25
2,5 DMPI-HSA	81	9	27
3,4-DMPI-HSA	73	8	22
KLH	0	0	0
2,4 TDI-KLH	84	–	–
2,6 TDI-KLH	82	–	–

[1] Assessment of protein mass shift using MALDI-TOF-MS: Spectra were produced on a Ciphergen PBS-IIc (Bio-Rad, Hercules, CA) linear time-of-flight mass spectrometer. Mass spectra were acquired at the threshold for ion production (laser step 140–150). Samples were prepared as dried droplets in 5 mg/mL alpha-cyano-4-hydroxycinnamic acid on gold-plated stainless steel sample plates for analysis. The spectra were externally calibrated with a protein mixture covering the molecular weight range between 10 and 66 kDa. Composite spectra were the average of 75 individual mass spectra from 15 positions across the sample deposit. The number of isocyanate moieties/protein was determined by measuring the upward mass shifts vs. the respective unmodified protein. Refer to Table 2 for chemical names and structures of the haptens.

2.10. Western blot analysis of mAbs cross-reactivity

One μg per lane of each TDI conjugate was separated by SDS-PAGE using a 7.5% polyacrylaminde gel and transferred over-night at 15 mA to a nitrocellulose membrane (0.2 μm, Biorad Hercules, CA). The membrane was blocked with 3% BSA for 1 h and reacted with a 1/10 dilution of desired mAb containing culture supernatant fluid for 1 h. After washing, the blot was reacted for 1 h with a 1/5000 dilution of alkaline-phosphate (AP) labeled anti-IgG H&L (Promega Madison, WI). Bound immune complexes were visualized using the AP substrate nitroblue tetrazolium and bromo-chloro-indolyl phosphate (NBT/BCIP Promega Madison, WI). Color was allowed to develop for 5 min and the reaction terminated by washing the membranes in distilled water.

2.11. Dot blot analysis

Dot blot analysis was carried out to evaluate the mAb reactivity towards native and denatured protein conjugates for 35 different conjugates. Native conjugates and carrier proteins (20 ng/spot) were spotted onto a nitrocellulose membrane (0.2 μm, BioRad) and allowed to dry overnight. The dot blots were repeated using denatured conjugates and carrier proteins that were treated with 2-mercaptoethanol (final concentration 25 mL/L) at 100 °C for 10 min prior to spotting. All the other steps were identical to the Western blot protocol with regard to incubation times and color development.

2.12. Experimental TDI Cell exposure systems

Mouse serum albumin (MSA, 2 mg/ml, Sigma,MO) or live A549 and BEAS-2B cells (ATCC) and fully differentiated human airway epithelial cultures (EpiAirway, Mattek Corperation, Ashland, MD) were exposed to 80/20 vapor 2,4/2,6 TDI using an air–liquid interface VITROCELL® exposure system (VITROCELL Systems, Inc. Waldkirch, Germany). Cells were seeded on Transwell® culture inserts and grown at 37 °C/5% CO_2 to confluence prior to exposure. A vapor atmosphere of 50 ppb TDI was generated using our previously described animal exposure system (Johnson et al., 2007). Briefly, neat TDI was injected into a heated air stream using a microprocessor controlled syringe pump and then mixed with the appropriate volume of dilution air to achieve 50 ppb within the exposure chamber. Concentration was monitored in real time using Remote Intelligent Sensor (RIS) Area Monitors (Scott Instruments, Monroe NC). The TDI vapor was drawn across the surface of the MSA solution or cells at the air–liquid interface using a vacuum pump. The air flow rate (static open air, 20, 100 and 200 ml/min) and the exposure time (1, 2 and 4 h) were varied to determine their impact on protein conjugation. The exposure system was maintained at 37 °C throughout the entire exposure using a circulating water bath. Immediately following exposure, the MSA solution was aliquoted and frozen at −80 °C until analysis. Total protein from cell cultures were harvested using the T-Per total protein extraction reagent (Thermo Scientific/Pierce, Rockford IL) according to the manufacturers' protocol and lysates were frozen at −80 °C until analysis.

2.13. Western blot detection of TDI-protein conjugation in cell extracts

One μg of TDI-MSA conjugate (positive control) prepared in solution as per Johnson et al. (Johnson et al., 2007) and 10 μg of MSA or total cell lysate were separated by electrophoresis using a 7.5% SDS-PAGE gel for 1 h and then transferred for 1 h at 100 V to a Polyvinylidene Fluoride (PVDF) membrane at 4 °C. The membrane was blocked with Odyssey Blocking Buffer® (Li-Cor Bioscience, Lincoln NE) for 1 h at room temperature and then incubated with various isocyanate-specific monoclonal antibodies overnight at 4 °C. After washing, the blot was incubated with goat anti-mouse IgG secondary antibody tagged with IRDye® 800CW (Li-Cor Bioscience, Lincoln NE) for 1 h with a 1:15,000 dilution. The proteins were detected using an Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln NE)

3. Results

3.1. Characterization of the dNCO conjugates

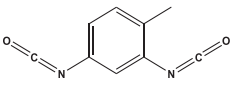
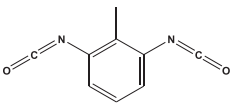
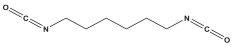
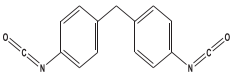
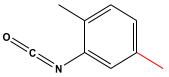
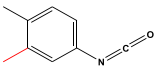
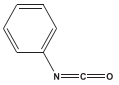
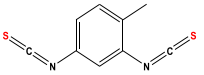
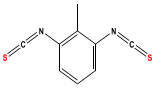
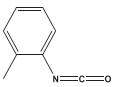
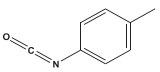
A panel of characterized mono- and diisocyanate, and diisothiocyanate conjugated proteins was produced for subsequent use in mAb binding specificity characterization. The extent of conjugation to protein was analyzed by two different methods (Table 1). Assessment by the TNBS dye indicated extensive chemical conjugation to the proteins in most cases;. Conjugates to keratin and lysozyme, however, gave negative results. The TNBS reagent assesses chemical adduction to primary amines of amino acids (Takahashi et al., 1984) on the surface of a (non-denatured) protein. A negative result for dNCO or diisothiocyanate (dNCS) binding to a protein may reflect binding of one NCO to the primary amine with hydrolysis of the other NCO functional group to an amine giving no net change in TNBS reactivity.

Additional analyses were performed using matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF-MS). For a molar ratio of 1:40 HSA:dNCO, the TNBS assay indicated 10 bound TDI adducts, which is in close agreement with the 11 surface accessible amine groups on HSA. However, the shift in mass observed by MALDI-TOF-MS indicated that 23 TDI moieties were bound to HSA. The determination of bound dNCOs using MALDI-TOF-MS was calculated by mass difference. The observed *m/z* for control HSA was 66,679 u. The observed *m/z* for 2,4 TDI-HSA was 70,743 u indicating an average of 23 dNCO moles/mole HSA, while that for 2,6 TDI-HSA indicated a ratio of 43 dNCO mol/mol HSA (Table 1). KLH conjugates were not amenable to MALDI-TOF-MS analyses as KLH is highly polymerized with a very wide mass range. Observation of a peak at approximately *m/z* 120,000 u in the MALDI-TOF-MS mass spectra of TDI-HSA conjugates also suggested formation of a TDI-bound protein dimer of the form $[2\,M + TDI + H]^+$. This is most likely due to dNCO cross-linking 2 protein molecules. The dimethyl phenyl isocyanate conjugates showed increased isocynate binding despite the lack of a second isocyanate group for polymerization. Recent protein sequencing data from our laboratory using reversed phase HPLC-MS/MS of 3,4 DMPI HSA conjugates indicates 17 binding sites on HSA, sixteen lysines and the N-terminal aspartic acid (Hettick et al., 2010).

3.2. Monoclonal antibody characterization

A total of 49 hybridomas were produced. Antibody concentrations in the culture supernatant fluids ranged from 0.160 μg/mL to 169 μg/mL with an average concentration of 52 μg/mL. Isotyping showed 29 hybridomas secreted IgG_1, 14 IgG_{2a}, 4 IgG_{2b}, and 2 IgG_3 antibodies. All hybridomas produced κ light chains. Initially, mAbs were screened by ELISA using very high mAb titers with TDI-HSA ELISA OD values off-scale to observe any reactivity toward other NCO, dNCO and dNCS conjugates. Monoclonal antibodies were divided into seven major groups based on similar patterns of reactivity to the protein conjugates and reassayed at lower mAb titers (Table 2). All ELISA absorbance values have been corrected for non-specific binding.

Table 2
Results of the ELISA analysis of 7 monoclonal antibodies against toluene diisocyanate conjugated proteins[2].

Chemical Name	Structure	Hapten-Protein (4 μg/mL)	ELISA readings (OD_{405nm}) and Dot blot scoring (+++)						
			2E5	60G2	62G5	79G7	16C6	32B6	59E9
2,4-toluene diisocyanate		2.4 TDI-HSA	2.70	3.57	2.88	3.20	2.54	2.65	0
			+++	+++	+++	+++	+++	+++	0
		2.4 TDI-KLH	2.29	3.74	3.88	3.69	2.85	2.65	0
			+++	+++	+++	+++	+++	+++	0
		2.4 TDI-MSA	2.57	3.61	3.21	3.62	2.45	2.40	0
			+++	+++	+++	+++	++	+++	0
		2.4 TDI-keratin	1.08	3.11	0.55	1.31	0	2.01	0
			++	+++	++	+++	0	+	0
2,6-toluene diisocyanate		2.6 TDI-HSA	0	3.66	3.77	2.60	3.13	3.68	3.96
			0	+++	+++	++	+++	+++	+++
		2.6 TDI-KLH	0	3.44	3.92	3.23	2.19	3.01	3.03
			0	+++	+++	+++	++	++	0
		2.6 TDI-MSA	0	0.86	1.72	0.50	0.30	0.66	0
			0	++	+++	++		+++	+
		2.6 TDI-keratin	0	0	2.03	0.42	2.18	3.16	0
			0	++	+++	+	0	+++	+
		2,6 TDI- collagen	0	0	1.75	0	0	0	0
			0	0	0	0	0	0	0
hexamethylene diisocyanate		HDI-HSA	0	0.21	0	0.75	0	0	0
			0	+	0	+	0	0	0
4,4-methylene diphenyl diisocyanate		MDI-HSA	0	0.66	0	0.21	0	0	0
			0	++	0	+	0	0	0
80/20 mix 2,4;2.6 TDI		2,4;2,6 TDI-HSA							
			3.85	3.81	3.26	3.56	3.78	2.80	1.13
			+++	+++	+++	+++	+++	+++	+
2,5-dimethyl phenylisocyanate		2,5 DMPI-HSA	0	0.64	2.15	3.2	0	0.62	0
			0	+	+++	+++	0	+	0
3,4-dimethyl phenylisocyanate		3,4 DMPI-HSA	0	0.27	0.77	0.61	0	0	0
			0	+	+++	+++	0	+	0
Phenyl isocyanate		PI-HSA	0	0	0	0	0	0	0
			0	+	0	0	0	0	0
2,4-toluene diisothiocyanate		2,4 TITC-HSA	0	0	0	0	0	0	0
			0	0	0	0	0	0	0
2,6-toluene diisothiocyanate		2,6 TITC-HSA	0	0	0	0	0	0	0
			0	0	0	0	0	0	0
2-toluene isocyanate		OTI-HSA	0	0	3.65	3.8	0	0	0
			0	0	+++	++	0	0	0
4-toluene isocyanate		PTI-HSA	0	0	0	0	0	0	0
			0	0	0	0	0	0	0

[2] The results represent the mean OD_{405} of 4 ELISA well repeats which were corrected by subtracting the average OD_{405} of 4 ELISA background control wells. Assay background controls were processed in parallel but contained HSA as the coating antigen. Positive values were considered to be 2.5 times the OD_{405} value of HSA or conjugating protein like MSA. A zero value indicates that the OD_{405} or visual dot were insignificant. 2E5, 60G2, 62G5 and 76G7 are from 2,4 TDI-KLH immunized mice, whilst 16C6, 32B6 and 59E5 are from 2,6 TDI-KLH immunized mice. 2,4/2,6 TDI-lysozyme, 2,4 TDI-collagen, data not shown as they had insignificant reactivities. Note the abbreviations for each chemical hapten are given in the Hapten-Protein column.
Dot Blot Key: +++ Strong reaction, ++ Moderate reaction, +Weak reaction.

Eight mAbs reacted only with 2,4 TDI-HSA, and 1 mAb reacted only to 2,6 TDI-HSA. Table 2 shows the ELISA results for seven mAbs, each representing one group of mAbs with similar reactivity as well as the dot ELISA semi-quantitative results for mAbs tested by dot ELISA. The 2E5 mAb group only reacted with 2,4 TDI-conjugated proteins, while 59E5 group only reacted with 2,6 TDI-conjugated proteins. The rest of the mAbs reacted with 2,4/2,6 TDI-HSA, 2,4/2,6 TDI-KLH, and 2,4/2,6 TDI-mouse serum albumin (MSA). None of the mAbs reacted with 2,4/2,6 TDI-lysozyme, but good reactivity toward TDI-keratin was observed, (data not shown). The 60G2 and 79G6 mAb groups reacted (but to a lesser extent) with HDI-HSA and MDI-HSA conjugates. The mAbs displayed similar reactivity by Dot blot assays. There were no qualitative differences between the native and denatured dot blots; however denatured proteins resulted in higher assay sensitivity (data not shown). The mAbs were also tested against a commercial product containing an 80/20 mixture of 2,4/2,6 TDI and MDI (The Gorilla Glue Company, Cincinnati, OH) conjugated to HSA. All the MDI-HSA reactive mAbs reacted with the commercial Gorilla glue-HSA conjugate in the ELISA format. Interestingly, mAb 60G2 reacted with the Gorilla glue (containing both monomeric and polymeric MDI) conjugate in the ELISA format, but not in the dot blots (data not shown).

3.3. Determination of affinity constant (K_a) by ELISA

The affinities of select mAbs from each of the reactivity patterns were determined by ELISA using 4 concentrations of antigen and constant mAb concentrations as described in Materials and Methods section of this manuscript. The calculated average K_a for select mAbs ranged from a K_a of $2.21\times10^{7}M^{-1}$ (mAb 79G7) to the highest K_a value of $1.07\times10^{10}M^{-1}$ (mAb 59E5). K_as for individual mAbs are not shown.

3.4. Western blot mAb binding characterization of TDI conjugated proteins and experimental TDI cell exposure culture extracts

The method of introduction of the dNCO to the protein can greatly affect the nature of the conjugate formed and potentially influence antibody recognition. After adopting a slow dNCO (in acetone) infusion method, the pattern of reactivity of TDI conjugated albumin was qualitatively compared to protein conjugated by passive diffusion or active TDI vapor flow across the albumin/PBS solution using Western Blot analyses (Fig. 1A). Extent of TDI conjugation to albumin was both time and air flow (across the albumin/PBS surface) dependent. Fifty ppb TDI vapor at 200 mL/min for 1 h produced antigenic TDI haptenated albumin with bands below, at, and above the level where unconjugated albumin migrates (Fig. 1A and C). The specificity of the reactivity of select mAbs was also analyzed using Western blot, in addition to both ELISA and Dot blot. As shown in Fig. 2, antibodies from clone 2E5 were specific to 2,4 TDI-HSA. 60G2 reacted with both 2,4 and 2,6 TDI-HSA. The mAb 60G2 also had weak reactivity to HDI and MDI bound HSAs probably through specific recognition of the urea bond linkage formed by reacting the NCO to the protein amine.

A549 cells grown at air liquid interface, and mouse serum albumin (MSA) (2 mg/ml) were exposed to 50 ppb of TDI vapor

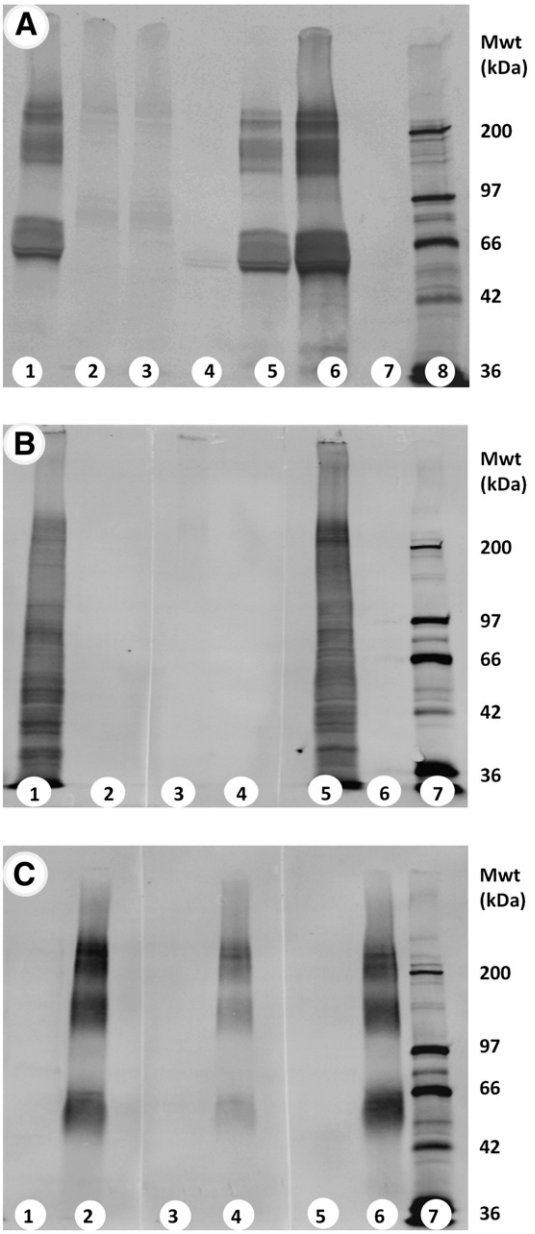


Fig. 1. A: Western Blot of TDI Vapor Exposed Mouse Albumin (MSA) and A549 Human Lung Epithelial Cells, developed with anti-TDI-protein mAb 60G2: Dark bands on MSA and cell extract lanes indicate binding of mAb. Lane 1: TDI-MSA (TDI passive diffusion, 1 h), lane 2 and 3: A549 Cells (TDI passive diffusion, 1 h), lane 4: TDI-MSA (TDI vapor flow rate = 20 mL/min, 1 h), lane 5: TDI-MSA (TDI vapor flow rate = 200 mL/min, 1 h), lane 6: TDI-MSA(TDI passive diffusion, 4 h), lane 7: Unexposed MSA (negative control), lane 8: DyLight® Molecular weight (Mwt) marker. B: Western Blot of TDI vapor exposed human airway epithelial cell model (EpiAirway) using mAbs reactive with 2,4-TDI, 2,6-TDI or 2,4/2,6-TDI conjugates. Lane 1: TDI exposed EpiAirway stained with mAb 79G7, Lane 2: filtered air exposed EpiAirway stained with mAb 79G7, Lane 3: TDI exposed EpiAirway stained with mAb 2E5, Lane 4: filtered air exposed EpiAirway stained with mAb 2E5, Lane 5: TDI exposed EpiAirway stained with mAb 58E9, Lane 6: filtered air exposed EpiAirway stained with 58E9, Lane 7: DyLight® Mwt marker. C: Western Blot of TDI-MSA conjugate (liquid infusion of TDI produced TDI-MSA conjugate) using the three mAbs from part B demonstrating reactivity to TDI-MSA. Lane 1: blank with mAb 79G7, Lane 2: TDI-MSA with mAb 79G7, Lane 3: blank with mAb 2E5, Lane 4: TDI-MSA with mAb 2E5, Lane 5: blank with mAb 58E9, Lane 6: TDI-MSA with mAb 58E9, Lane 7: DyLight® Mwt marker.

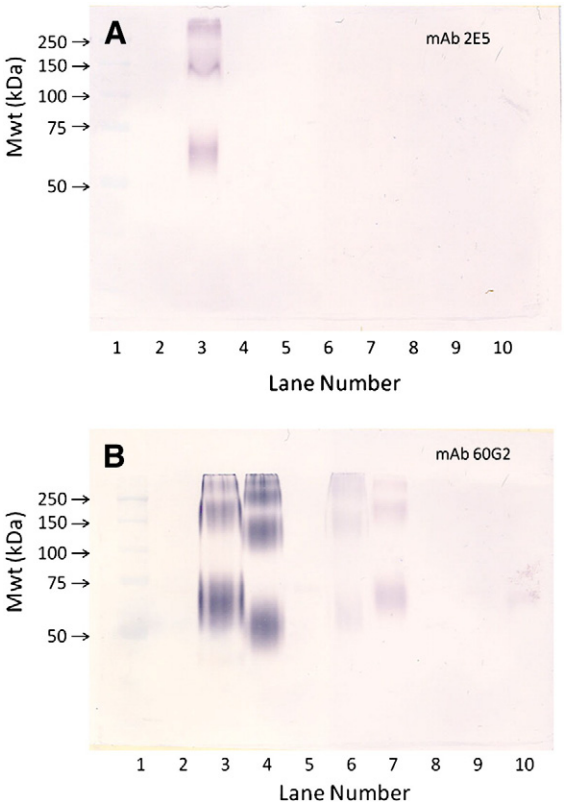


Fig. 2. Western blots of (2A) mAb 2E5 reactivity and (2B) mAb 60G2 reactivity. mAb 2E5 displays highly specific reactivity toward 2,4 TDI-HSA and 60G2 reacts with multiple mono and diisocyanates haptenated HSAs. Lane 1: MW markers (Mwt), lane 2: HSA, lane 3: 2,4 TDI-HSA, lane 4: 2,6 TDI-HSA, lane 5: phenyl isocyanate (PI)-HSA, lane 6: methylene diphenyldiisocyanate (MDI)-HSA, lane 7: hexamethylene diisocyanate (HDI)-HSA, lane 8: o-toluene isocyanate (OTI)-HSA, lane 9: p-toluene isocyanate (PTI)-HSA and lane 10: 2,4 toluene diisothiocyanate (TITC)-HSA.

using various times and diffusion rates. A549 cells (TDI passive diffusion, 1 h) grown at the air–liquid interface as well as 2 mg/ml solution of MSA in PBS (TDI at vapor flow rates of 20 and 200 mL/min for 1 h or passive diffusion of TDI for 1 and 4 h) were exposed to 50 ppb of TDI vapor. Cell protein extracts were subjected to gel electrophoresis and immunoblotting and compared to the TDI-MSA conjugates and unconjugated MSA (negative control). Fig. 1A shows that the mAb 60G2 reacts with multiple TDI vapor exposed cellular lung proteins (lanes 2 and 3), and TDI vapor conjugated MSA in an exposure time and flow rate dependent manner. BEAS-2B cells were also exposed to TDI and multiple bands were also observed after staining with 60G2 mAb (data not shown). Lanes 1, 5 and 6 show a strong band in the 70 kDa range after TDI exposure of MSA by passive diffusion. As was expected, higher exposure flow rates (200 mL/min, lane 5) resulted in more intense staining than the lower exposure flow rates (20 mL/min, lane 4). Importantly, multiple bands were observed in A549 cells exposed by passive diffusion indicating TDI conjugation to multiple cellular proteins.

Next, fully differentiated human airway epithelial cell air–liquid interface cultures (EpiAirway) were exposed to 50 ppb TDI vapor for 2 h at a flow rate 100 ml/min. Total cell protein was extracted and subjected to Western blotting using 2,4 TDI specific (2E5), and 2,4/2,6 TDI specific (79G7 and 58E9) antibodies (Fig. 1B). Monoclonal antibodies 79G7 and 58E9 are IgG2a and IgG1, respectively. They have similar reactivity patterns. The mAbs specific for both 2,4 TDI and 2,6 TDI conjugated proteins (mAb 79G7 and 58E9) showed strong staining of multiple molecular weight bands in the airway cultures that were treated with TDI, but no reactivity was observed for cultures exposed to filtered air alone. However, the 2,4 TDI conjugate specific mAb (2E5) did not stain any proteins from the TDI or air exposed cultures. It is important to note that all three mAbs were tested on the same protein extracts. To demonstrate that the 2,4 TDI mAb was capable of binding TDI-conjugated protein, a gel was run in which all three mAbs were used to detect the TDI-MSA positive control. Fig. 1C clearly shows that all three mAbs are capable of binding to TDI-MSA with similar intensity.

4. Discussion

In a previous study (Ruwona et al., 2010), we sensitized mice by TDI-vapor exposure to generate monoclonal antibodies. Only TDI-specific IgM mAbs, however, were obtained, despite the mice having significant sera titers of TDI-specific IgG. Most of the IgM mAbs obtained recognized the TDI conjugate in relation to albumin resulting in significant reactivity toward unconjugated albumin. This may have been inherent to the nature of IgMs or the predominance of TDI albumin as the major circulating conjugated form (Lind et al., 1997). As the true immunogenic form of TDI-conjugated protein is not known, it was felt that this approach greatly favored haptenated albumin and possibly decreased the potential for obtaining a useful panel of TDI-mAbs of varying affinities.

The allergenic dNCO-haptenated proteins in skin and lung following occupational dNCO exposure are unknown. While *in vitro* conjugated albumins have been demonstrated to be antigens and the degree of haptenation important for antigenicity, it remains to be determined how well these reflect the haptenated epitopes that are formed *in vivo*. The aims of the present approach were to optimize a panel of anti-TDI-protein IgG mAbs that would be carrier protein-independent, and able to distinguish between different aromatic and aliphatic diisocyanates, diisothiocyanates and monoisocyanates. Since TDI quickly hydrolyzes in aqueous environments or reacts with nucleophiles in the body, specific antibody production to unreacted dNCO is highly improbable and the antibodies would be expected to react to conjugated forms of dNCOs. In addition, if antibodies to dNCOs could be formed, the dNCOs would react directly with nucleophilic moieties on the antibody resulting in dNCO-mAb conjugation products as opposed to directly binding to free dNCO.

Identification and characterization of specific dNCO haptenated proteins from isocyanate exposed workers is an important step for developing the best diagnostic assays that are sensitive enough to detect exposure as well as disease development. Diisocyanates may react with multiple nucleophilic moieties within a protein, although the gamma amine on lysines is consistently found as a target (Wisnewski et al., 2010). TNBS and MALDI-TOF-MS analyses employed in the present study resulted in discordant results as to the estimated number of isocyanates bound. This may have been due to the bifunctional nature of dNCOs causing extensive cross linking and polymerization of the dNCO. The present results illustrate some of the pitfalls with the TNBS method including measurement of conjugation to the hydrophobic keratin,

poor accuracy for measurement of binding to basic proteins e.g. lysozyme, (Goodwin and Choi, 1970), and to haptens that can hydrolyse to an amine . Therefore, we used MALDI-TOF MS, a technique capable of determining more precisely the number of conjugated TDI molecules to further characterize the resulting TDI-protein haptens. However, MALDI-TOF-MS analysis provides only the mass-to-charge ratio of analyte; it provides no information with respect to binding sites, or degree of polymerization. Campo et al. (Campo et al., 2007) compared HDI conjugates produced by various research groups using different conjugation techniques with results ranging from 0.3 to 33 dNCO/HSA, in good agreement with the range of 0 to 43 dNCO/HSA determined in this study by mass spectrometry. Collectively our results and those of other investigators (Wisnewski and Redlich, 2001; Ott et al., 2007) demonstrate the difficulty in characterizing and standardizing dNCO-protein conjugates, even with consistent conjugation reaction ratios (Wisnewski, 2007). More advanced techniques including quadrupole time-of-flight tandem mass spectroscopy (qTOF-MS/MS) are now being explored (Hettick et al., 2009; Wisnewski et al., 2010). Despite negative TNBS results for TDI conjugation to both collagen and keratin, both conjugates could be by ELISA (Table 2) suggesting that the mAbs are exquisitely sensitive at detecting TDI haptens.

The majority of mAbs produced cross-reacted with multiple dNCO-protein conjugates, but mAbs specific for 2,4 TDI- or 2,6 TDI- proteins were also generated (Table 2). In general, mAbs that recognized both 2,4 and 2,6 TDI-HSA, displayed stronger recognition to mono-NCO haptenated proteins, particularly when the NCO was in the ortho position relative to the tolyl group. This was demonstrated by the following order of binding affinities with 2,3 DMPI >3,4 DMPI and OTI>>PTI or PI from the ELISAs . Antibody recognition was greatly reduced when one NCO was removed vs. substituted (by a methyl group). In the ELISA format, most mAbs were able to discriminate between isocyanate and isothiocyanate conjugates (i.e. between urea and thiourea linkage) and also between aromatic and aliphatic dNCOs conjugated to HSA. There was little dependence on immuno-assay format since the dot blots and the ELISA were in good agreement. Greater mAb reactivity was observed when using denatured protein conjugates in the dot blots possibly due to the exposure of cryptic TDI epitopes by unfolding the protein. This is in agreement with our earlier report with the TDI specific IgM mAb reactivity (Ruwona et al., 2010). In Western blots we observed extensive polymerization of the dNCO-HSA conjugates showing reactivity at 150 kDa and above. Antibody reactivity indicating molecular mass less than that of native HSA are most likely attributable to intramolecular TDI cross-linking preventing complete protein denaturation resulting in faster migration through the gel.

The affinity constant (K_a) of mAb is an important parameter for the application of mAbs (Beatty et al., 1987). High affinity mAb increase the specificity and sensitivity of immunoassays and may be more useful in the development of a diagnostic method for detection of *in vivo* conjugated proteins. The mAbs produced in this study have high affinity $K_a > 10^7$ for 2,4/2,6 TDI conjugated HSA and are fit for developing tests for detection of TDI bound proteins. The mAb with reactivity only to 2,6 TDI-protein (59E5), also had the highest affinity, whilst the more promiscuous mAb, 79G7, had the lowest affinity to 2,4 TDI-HSA. As expected, the more discriminating mAbs had greater affinities for their respective target TDI-conjugates than the less discriminating mAbs.

An important step in characterizing these mAbs is demonstrating that they are capable of binding to conjugated proteins that were derived from cells exposed to TDI vapor. A549 and BEAS-2B human airway epithelial cells were exposed to TDI vapor and mAb 60G2 was used as a detection of TDI-conjugated proteins in total cell protein Western blots. This mAb was selected since it displayed a broader range of reactivity (Table 2). Greater reactivity was observed with higher exposure flow rates or higher exposure time using TDI-haptenated MSA. The immune-blot pattern of mAb reactivity demonstrates that the TDI vapor conjugated MSA was similar to that produced by the controlled infusion conjugation method employed for mAb production and characterization. Significantly, detection of TDI conjugated proteins isolated from A549 and BEAS-2B cells exposed to only 50 ppb TDI was demonstrated by immune-blot analysis. This analysis demonstrates the physiological relevance of the anti-TDI-protein mAbs. The identity of the specific cellular proteins bound by TDI has not yet been demonstrated, however, the Western blot for A549 and BEAS-2B cell extracts suggests that a variety of cellular proteins reacted with TDI vapor.

The TDI used for *in vitro* experiments was a commercial grade 80/20 mix of 2,4/2,6 TDI. In order to determine the specificity of our classes of mAbs, we examined the ability of 2,4 TDI hapten specific, and 2,4/2,6 TDI hapten non-discriminating mAbs using the same protein extracts from filtered air and TDI (80/20 mix) exposed fully differentiated air–liquid interface airway epithelial cultures. Interestingly, the 2,4 TDI conjugate specific mAb was unable to detect any proteins. The 2,4 TDI-reactive mAb that we selected showed almost exclusive specificity for 2,4 TDI-HSA when characterized with our panel of conjugates. Therefore, it may only be capable of recognizing TDI when conjugated to albumin as shown by ELISA and immune-blot in the current study, or that the specific haptenated epitope in the cultured cells recognized by the 2,4 TDI specific mAb was of very low abundance.

In summary, a significant gap in the understanding of dNCO-induced allergic reactions is the identity of *in vivo* targets for dNCO haptenation. We have successfully developed a panel of high affinity monoclonal antibodies that recognize dNCO-protein adducts from 2,4 or 2,6 TDI-KLH immunized mice and 2,4 or 2,6 TDI-HSA screened hybridomas. The specificity of these antibodies was characterized by ELISA, Western blot, and dot blot analysis. The potential utility as tools for the identification of endogenous dNCO-modified proteins and the characterization of disease-relevant chemical linkages was demonstrated using our mAb characterization and cell culture studies. Development of this mAb panel represents an important contribution that will advance our understanding of dNCO exposure and disease in exposed workers.

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References

Beatty, J.D., Beatty, B.G., Vlahos, W.G., 1987. Measurement of monoclonal-antibody affinity by noncompetitive enzyme-immunoassay. J. Immunol. Methods 100, 173.

Campo, P., Wisnewski, A.V., Lummus, Z., Cartier, A., Malo, J.L., Boulet, L.P., Bernstein, D.I., 2007. Diisocyanate conjugate and immunoassay characteristics influence detection of specific antibodies in HDI-exposed workers. Clin. Exp. Allergy 37, 1095.

Chappelle, A.H., Shiotsuka, R.N., Bartels, M.J., 2002. Some Limitations in the Use of Urine Biomonitoring for the Measurement of TDI Exposure. In: Lasage, J., DeGraff, D.I., Danchik, R.S. (Eds.), Isocyanates: Sampling, Analysis and Health Effects, ASTM STP 1408. American Society for Testing Materials, West Conshohocken, PA, p. 64.

Dalene, M., Skarping, G., Tinnerberg, H., 1994. Biological monitoring of hexamethylene diisocyanate by determination of 1,6-hexamethylene diamine as the trifluoroethyl chloroformate derivative using capillary gas chromatography with thermoionic and selective-ion monitoring. J. Chromatogr. B Biomed. Appl. 656, 319.

Goodwin, J.F., Choi, S.Y., 1970. Quantification of protein solutions with trinitrobenzenesulfonic acid. Clin. Chem. 16, 24.

Harlow, E., Lane, D., 1988. Antibodies-A Laboratory Manual. Cold Spring Harbor, NY.

Hettick, J.M., Ruwona, T.B., Siegel, P.D., 2009. Structural elucidation of isocyanate-peptide adducts using tandem mass spectrometry. J. Am. Soc. Mass Spectrom. 20, 1567.

Hettick, J.M., Ruwona, T.B., Siegel, P.D., 2010. Determination of the Binding Sites of Dimethylphenylisocyanate on Human Serum Albumin. 57th ASMS Conference on Mass Spectrometry and Allied Topics, p. 4.

Hur, G.Y., Koh, D.H., Choi, G.S., Park, H.J., Choi, S.J., Ye, Y.M., Kim, K.S., Park, H.S., 2008. Clinical and immunologic findings of methylene diphenyl diisocyanate-induced occupational asthma in a car upholstery factory. Clin. Exp. Allergy 38, 586.

Johannesson, G., Sennbro, C.J., Willix, P., Lindh, C.H., Jonsson, B.A., 2004. Identification and characterisation of adducts between serum albumin and 4,4'-methylenediphenyl diisocyanate (MDI) in human plasma. Arch. Toxicol. 78, 378.

Johnson, V.J., Yucesoy, B., Reynolds, J.S., Fluharty, K., Wang, W., Richardson, D., Luster, M.I., 2007. Inhalation of toluene diisocyanate vapor induces allergic rhinitis in mice. J. Immunol. 179, 1864.

Karol, M.H., 1986. Respiratory Effects of Inhaled Isocyanates. CRC Crit. Rev. Toxicol. 16, 349.

Lange, R.W., Lantz, R.C., Stolz, D.B., Watkins, S.C., Sundareshan, P., Lemus, R., Karol, M.H., 1999. Toluene diisocyanate colocalizes with tubulin on cilia of differentiated human airway epithelial cells. Toxicol. Sci. 50, 64.

Lind, P., Dalene, M., Lindstrom, V., Grubb, A., Skarping, G., 1997. Albumin adducts in plasma from workers exposed to toluene diisocyanate. Analyst 122, 151.

Mraz, J., Bouskova, S., 1999. 2,4-toluenediisocyanate and hexamethylene-diisocyanate adducts with blood proteins: assessment of reactivity of amino acid residues in vitro. Chem. Biol. Interact. 117, 173.

Ott, M.G., Jolly, A.T., Burkert, A.L., Brown, W.E., 2007. Issues in diisocyanate antibody testing. Crit. Rev. Toxicol. 37, 567.

Redlich, C.A., Karol, M.H., 2002. Diisocyanate asthma: clinical aspects and immunopathogenesis. Int. Immunopharmacol. 2, 213.

Ruwona, T.B., Johnson, V.J., Schmechel, D., Simoyi, R.H., Beezhold, D., Siegel, P.D., 2010. Monoclonal Antibodies Against Toluene Diisocyanate Haptenated Proteins from Vapor-Exposed Mice. Hybridoma 29, 221.

Sabbioni, G., Hartley, R., Schneider, S., 2001. Synthesis of adducts with amino acids as potential dosimeters for the biomonitoring of humans exposed to toluenediisocyanate. Chem. Res. Toxicol. 14, 1573.

Sabbioni, G., Dongari, N., Kumar, A., 2010. Determination of a new biomarker in subjects exposed to 4,4 '- methylenediphenyl diisocyanate. Biomarkers 15, 508.

Sashidhar, R.B., Capoor, A.K., Ramana, D., 1994. Quantitation of Epsilon-Amino Group Using Amino-Acids As Reference-Standards by Trinitrobenzene Sulfonic-Acid - A Simple Spectrophotometric Method for the Estimation of Hapten to Carrier Protein Ratio. J. Immunol. Methods 167, 121.

Schmechel, D., Simpson, J.P., Beezhold, D., Lewis, D.M., 2006. The development of species-specific immunodiagnostics for Stachybotrys chartarum: The role of cross-reactivity. J. Immunol. Methods 309, 150.

Sennbro, C.J., Lindh, C.H., Tinnerberg, H., Welinder, H., Littorin, M., Jonsson, B.A., 2004. Biological monitoring of exposure to toluene diisocyanate. Scand. J. Work Environ. Health 30, 371.

Takahashi, S., Yamamura, T., Kamo, M., Satake, K., 1984. Regeneration of Amino-Compounds from the 2,4,6-Trinitrophenyl Derivatives by Treatment with Hydrazine. Chem. Lett. 127.

Tinnerberg, H., Dalene, M., Skarping, G., 1997. Air and biological monitoring of toluene diisocyanate in a flexible foam plant. Am. Ind. Hyg. Assoc. J. 58, 229.

Wisnewski, A.V., 2007. Developments in laboratory diagnostics for isocyanate asthma. Curr. Opin. Allergy Clin. Immunol. 7, 138.

Wisnewski, A.V., Redlich, C.A., 2001. Recent developments in diisocyanate asthma. Curr. Opin. Allergy Clin. Immunol. 1, 169.

Wisnewski, A.V., Srivastava, R., Herick, C., Xu, L., Lemus, R., Cain, H., Magoski, N.M., Karol, M.H., Bottomly, K., Redlich, C.A., 2000. Identification of human lung and skin proteins conjugated with hexamethylene diisocyanate in vitro and in vivo. Am. J. Respir. Crit Care Med. 162, 2330.

Wisnewski, A.V., Liu, Q., Liu, J., Redlich, C.A., 2005. Glutathione protects human airway proteins and epithelial cells from isocyanates. Clin. Exp. Allergy 35, 352.

Wisnewski, A.V., Liu, J., Redlich, C.A., 2010. Antigenic changes in human albumin caused by reactivity with the occupational allergen diphenylmethane diisocyanate. Anal. Biochem. 400, 251.