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Production, Characterization and Possible Applications of Monoclonal Antibodies

Generated against Toluene Diisocyanate–conjugated Proteins

by

Tinashe Blessing Ruwona

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Chemistry

Dissertation Committee:
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ABSTRACT

Diisocyanates are very reactive low molecular weight chemicals that are widely used in the manufacture of polyurethane products. Diisocyanate exposure is one of the most commonly reported causes of occupational asthma. Although diisocyanates have been identified as causative agents of respiratory diseases, the specific mechanisms by which these diseases occur remain largely unknown.

Tandem mass spectrometry was used to unambiguously identify the binding site of isocyanates within four model peptides (Leu-enkephalin (Leu-enk, YGGFL), Angiotensin I (DRVYIHPFHL), Substance P-amide (RPKPQQFFGLM-NH₂), and Fibronectin-adhesion promoting peptide (FAPP, WQPPRARI)). In each case, isocyanates were observed to react to the N-terminus of the peptide. No evidence of side chain/isocyanate adduct formation exclusive of the N-terminus was observed. However, significant intra-molecular diisocyanate crosslinking between the N-terminal amine and a side chain amine group was observed for arginine, when located within two residues of the N-terminus. Addition of multiple isocyanates to the peptide occurs via polymerization at the N-terminus, rather than addition of multiple isocyanate molecules to varied residues within the peptide.

Toluene diisocyanate (TDI)-specific monoclonal antibodies (mAbs) with potential use in immunoassays for exposure and biomarker assessments were produced. A total of 59 unique mAbs were produced (29 IgG₁, 14 IgG_{2a}, 4 IgG_{2b}, 2 IgG₃ and 10 IgM) against 2,4 and 2,6 TDI bound protein. The reactivities of these mAbs were characterized by a solid phase indirect enzyme-linked immunosorbent assay (ELISA), Dot ELISA and Western immunoblot against various monoisocyanate, diisocyanate and dithioisocyanate protein conjugates. A subset of the mAbs were specific for 2,4 or 2,6 TDI-conjugated proteins only while others reacted to multiple dNCO conjugates including methylene diphenyl diisocyanate- and hexamethelene diisocyanate– human serum albumin . Western blot analyses demonstrated that some TDI conjugates form inter- and intra-molecular links resulting in multimers and a change in the electrophoretic mobility of the conjugate.

In general, 2,4/2,6 TDI reactive mAbs displayed (1) stronger recognition of monoisocyanate haptenated proteins when the isocyanate was in the ortho position relative to the tolyl group, and were able to discriminate between (2) isocyanate and isothiocyanate conjugates (i.e. between the urea and thiourea linkage); and (3) between aromatic and aliphatic diisocyanates. The mAbs produced were not carrier protein specific with estimated affinity constants toward toluene diisocyanate conjugated human serum albumin ranging from 2.21×10^7 to $1.07 \times 10^{10} \text{ M}^{-1}$ for IgG mAbs. Studies using TDI vapor exposed lung and epithelial cell lines suggest potential utility of these mAbs for both research and biomonitoring of isocyanate exposure.

To my mother and father -

I owe it to you...

And

To my wife -

Nothing is impossible...

All religions, arts and sciences are branches of the same tree. All these aspirations are directed toward ennobling man's life, lifting it from the sphere of mere physical existence and leading the individual towards freedom.
- Albert Einstein

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Scheme 3.1: The highly reactive nature of isocyanates

is highlighted in this scheme.

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LIST OF ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ACD	allergic contact dermatitis
AHSA	acetylated human serum albumin
AP	alkaline phosphatase
APC	antigen presenting cell
ATCC	American Type Culture Collection
CID	collision-induced dissociation
CSN	culture supernatant
DDI	distilled deionized
DMEM	Dulbecco's Modified Eagles Media
DMPI	dimethy phenyl isocyanate
DMSO	dimethyl sulfoxide
dNCO	diisocyanates
ELISA	enzyme linked Immunosorbent assay
ESI	electrospray ionization
HAT	hypoxanthine-aminopterin-thymidine
HDI	hexamethylene diisocyanate
HEPA	high efficiency particulate air

HGPRT ⁻	hypothanthine-guanine phosphoribosyltransferase deficient
HMDI	methylene bis-cyclohexylisocyanate
HPLC-Q-TOF	high performance liquid chromatographic- quadrapole-time of flight mass spectrometry
HSA	human serum albumin
IACUC	Institutional Animal Care and Use Committee
ICD	irritant contact dermatitis
IP	intraperitoneal
IPDI	isophorone diisocyanate
K _a	affinity constant
K _d	dissociation constant
KLH	keyhole limpet hemocyanin
LMW	low molecular weight
m/z	mass to charge ratio
mAbs	monoclonal antibodies
MALDI	matrix assisted laser desorption and ionization
MDI	methylene diphenyl diisocyanate
MHC1	major histocompatibility complex 1
MSA	mouse serum albumin
Mwt	molecular weight

NBT/BCIP	nitroblue tetrazolium and bromo-chloro-indolyl phosphate
NCO	isocyanate
NDI	naphthalene diisocyanate
NIOSH	National Institute for Occupational Safety and Health
OA	occupational asthma
OD	optical density
OTI	ortho toluene isocyanate
PBS	phosphate buffered saline
PBSTM	phosphate buffered saline tween milk
PEG	polyethylene glycol
PI	phenyl isocyanate
PVDF	polyvinylidene fluoride
RIS	remote intelligent sensor
RT	room temperature
SDS-PAGE	sodium dodecyl sulphate-poly acrylamide gel electrophoresis
TDA	toluene diamine
TDI	toluene diisocyanate
TIC	total ion content
TITC	toluene diisothiocyanate

TNBS	trinitrobenzenesulphonic acid
TOF	time of flight
UV/Vis	ultra violet/visible

RATIONALE

Diisocyanates (dNCOs) are used in the production of polyurethanes. The most common monomeric dNCOs are toluene diisocyanate (TDI), methylene diphenyldiisocyanate (MDI) and hexamethylene diisocyanate (HDI)^{1,2}. People exposed to isocyanates can develop a range of short-term health problems such as headaches, sore eyes, sore throat, difficulty in breathing and skin irritation. Isocyanate exposure can also lead to long-term asthma and dermatitis if a person becomes sensitized. The most commonly reported cause of occupational asthma (OA) is from exposure to dNCOs³, with a prevalence of diisocyanate asthma estimated at 5-15% of exposed workers² in the United States of America (USA). Leigh *et al*⁴ estimated that the medical cost of OA in the USA for 1999 at \$1.48 billion. This direct medical cost does not include the tremendous socio-economic indirect cost with lost productivity and family burden.

Over 200,000 workers are directly employed in the production and use of isocyanates, worldwide⁵. The applications of isocyanates encompass virtually all aspects of our lives from agriculture to transport and leisure. They are commonly used in paints, glues/binders and foams. Due to their widespread use in the general population from products commercially available at hardware stores like Gorilla Glue (The Gorilla Glue Company, Cincinnati, OH) and Great Stuff Form (The Dow Chemical Company, Midland, MI), actual rates of dNCO induced diseases and exposure may be under-reported⁵. Concern has also been expressed for potential dNCO exposure to the

general public through end product breakdown or leaching⁶.

Health effects of dNCO exposure have been a subject of extensive research in terms of both human and animal toxicological studies. Dose-dependent responses to higher levels of dNCO's include respiratory⁷, dermal and mucous membrane irritation⁸. Hypersensitivity reactions to dNCO's include allergic rhinitis⁹, asthma⁷, hypersensitivity pneumonitis^{10,11} and allergic contact dermatitis¹².

Covalent conjugation (haptentation) of diisocyanate to human proteins after exposure is commonly accepted as an important primary event in the development of diisocyanate-induced allergic sensitization and asthma¹³. The major dNCO adducts found in the blood are to hemoglobin and albumin¹³⁻¹⁶. TDI-conjugated lung proteins in a murine study were co-localized by immunochemical staining with tubulin, and actin, which suggest that these proteins may also be conjugated¹⁷. Other skin and lung proteins and peptides like keratin, glutathione and actin have been reported as HDI binding targets^{15,18}. The immunogenic protein form(s), that lead to sensitization and asthma in the occupational environment however, cannot be inferred from these studies. It can be concluded from these reports, that TDI binding, *in vivo*, does demonstrate selectivity with respect to target proteins, however, the ultimate antigenic protein(s), or all forms of chemical linkage(s) are not yet known¹⁹.

The aims of this thesis were to 1) use spectroscopic and bionalytical methods such as mass spectroscopy to elucidate isocyanate-protein chemical interaction; and 2) produce monoclonal antibodies (mAbs) that recognize TDI-bound proteins. These monoclonal antibodies can be used for immunohistochemistry and for biomarker immunoassays of dNCO exposure. In addition, they should make useful research tools in identifying specific dNCO-bound proteins following both dermal and inhalation exposures. We hypothesize that the antigenic specificity of the mAbs produced will provide insight into *in vivo* immunopathogenicity, however, this is beyond the scope of the present study.

CHAPTER 1

INTRODUCTION

1.1 Industrial and Commercial Uses of Isocyanates

The predominant use of diisocyanates is in the manufacture of polyurethane forms, elastomers and coatings²⁰. Other nonpolymer uses include insecticides, pesticides and herbicides²¹ for mono isocyanates such as methyl isocyanate²⁰. The most widely used compounds are diisocyanates, which contain two isocyanate groups, and polyisocyanates, which are usually derived from diisocyanates and may contain several isocyanate groups. The most commonly used diisocyanates include MDI (glues, hard forms, and plastics), TDI (flexible forms, elastomers and coatings), and HDI (car paints). Others include naphthalene diisocyanate (NDI), methylene bis-cyclohexylisocyanate (HMDI) (hydrogenated MDI), and isophorone diisocyanate (IPDI). Examples of widely used polyisocyanates include HDI biuret and HDI isocyanurate. In these applications, formation of a urethane link is the predominant reaction. For this reaction the most common isocyanate is TDI (known as Mondur TD-80, Nacconate, TDI, Voranate T-80, TDI-80, Rubinate TDI in commercial preparations), which is an 80:20 mixture of 2,4-TDI and 2,6-TDI²². Toluene diisocyanates are not known to occur as natural products. They are manufactured by the reaction of diaminotoluenes with phosgene^{8,21}. Toluene diisocyanates are reactive intermediates that are used in combination with polyether and polyester polyols to produce polyurethane products. The production of flexible polyurethane foams

represents the primary use of toluene diisocyanates (approximately 90% of the toluene supply)²⁰. Polyurethane coatings represent the second largest market for toluene diisocyanates. Toluene diisocyanates are also used in the production of polyurethane elastomeric casting systems, adhesives, sealants and other uses. TDI is one of the most common isocyanates employed in the manufacture of polyurethane foams, elastomers, and coatings. Foams are used in furniture, packaging and insulation as well as boat building. Flexible foams are made from TDI, whereas the rigid foams are made from the less volatile MDI⁸. Polyurethane coatings are used in leather, wire, tank linings, masonry, paints, floors and wood finishes. Elastomers are abrasion and solvent resistant, and are used in adhesives, coated fabrics, films, linings, clay pipe seals, and in abrasive wheels, and other mechanical items²⁰.

1.2 Health Effects of Diisocyanates

1.2.1 Routes of Exposure

The main route of exposure to TDI is through inhalation²³. Responses to TDI vary widely from mild irritation of the airways to more severe effects, including bronchospasm. Other routes of exposure include oral ingestion, eye contamination, and skin contamination⁸.

1.2.2 Toxic Effects

Respiratory effects are the primary toxicological manifestations of repeated exposure to diisocyanates⁸. Isocyanates are powerful irritants to the mucous membranes of the

eyes, gastrointestinal tract and respiratory system²⁴. Direct skin contact can also cause marked dermal inflammation. Diisocyanates can also sensitize workers, making them subject to allergic rhinitis, allergic contact dermatitis and asthma attacks upon re-exposure. Death from severe asthma in sensitized subjects has been reported⁷.

1.2.3 Diisocyanate Structure and Reactivity with Biological Molecules.

Figure 1.1 shows some of the major reactions that TDI can potentially undergo. Of particular importance is the adduct formation to biomolecules. Hydroxyl, amino, and sulfhydryl groups are all found in biological systems. TDI has the potential to react with any of these groups or water after entry into the body, and therefore it can be assumed that no unreacted diisocyanate circulates after absorption. Isocyanates can react with the following amino acids under physiological condition²⁵; the α -amino group of the N-terminal amino acids, the sulfhydryl group of cysteine, the hydroxyl groups of tyrosine and especially serine, the ϵ -amino group of lysine and the imidazole ring of histidine.

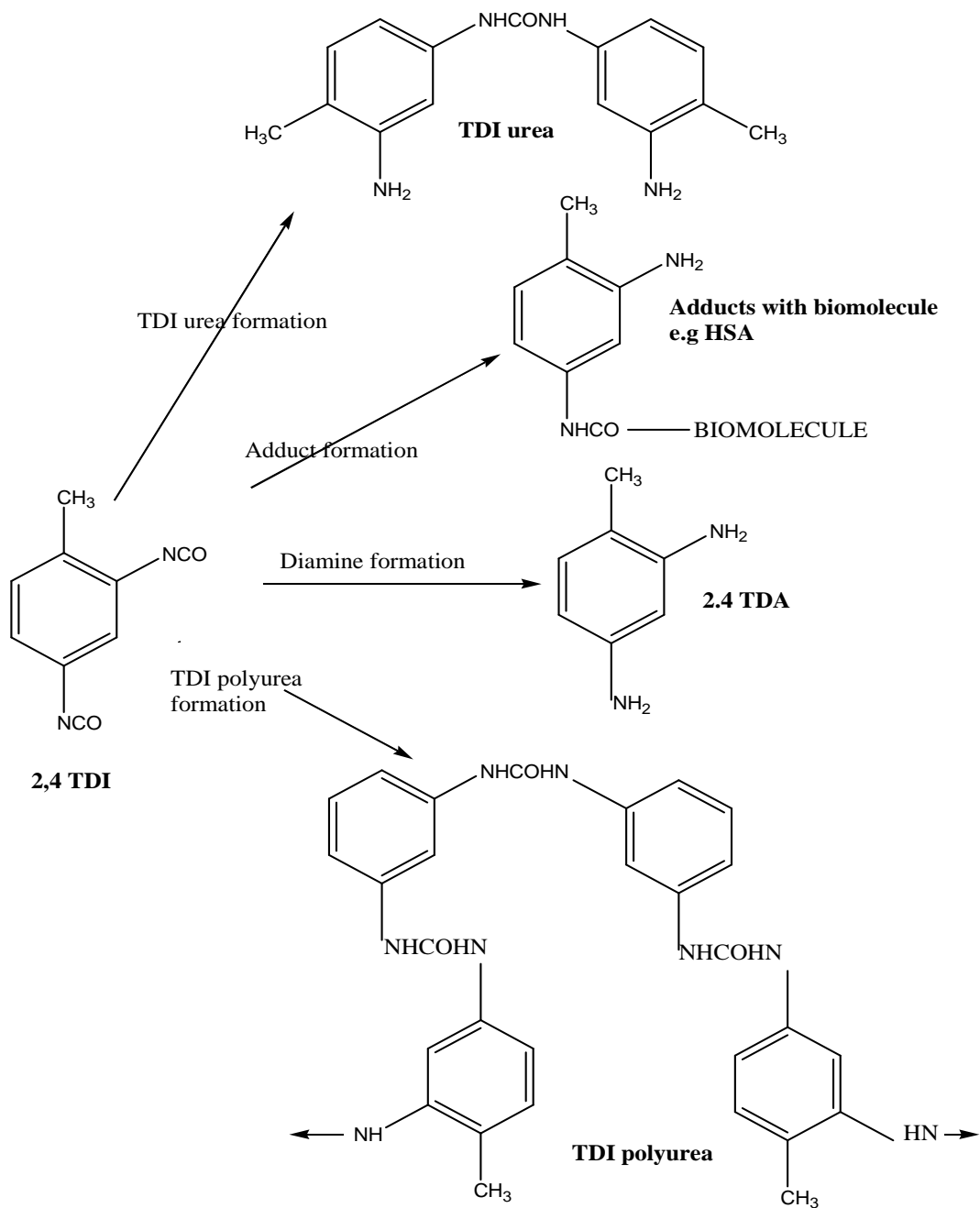


Figure 1.1: Competitive reaction pathways of TDI in biological systems ⁸.

The hydrolysis of TDI to give corresponding diamines is normally a minor reaction under conditions prevailing in the respiratory tract. At concentrations normally found in the work place, the predominant reactions are those involving biomolecules such as proteins. The extent to which a particular reaction takes place, if at all, depends on a number of parameters including:

- the physical form of diisocyanate (vapor, aerosol or liquid);
- the mode of exposure to diisocyanate (inhalation, ingestion or by skin contact, with or without solvent);
- the concentration of diisocyanate at the reaction site;
- the biological availability of the reactive molecules at the application site (different biological systems contain substance and structures which can significantly affect reaction pathways) and
- the prevailing biological pH.

1.2.4 Contact Dermatitis

Dermatitis may occur as a result of exposure to chemicals in the workplace. Irritant contact dermatitis (ICD) is the most common form of chemical induced dermatitis. It is a dose-dependent toxicity/non-immunologically mediated effect associated with a chemical's ability to react with skin components and damage the skin. Allergic contact dermatitis (ACD) is a T-lymphocyte mediated delayed (Type IV) hypersensitivity/immunological reaction²⁶. Examples of this type of reaction include a positive tuberculin skin test and the reaction to poison ivy. Both ICD and ACD can be

produced by dermal exposure to dNCOs²⁷. Diagnosis of DTH is usually confirmed by clinical dermal patch testing. Currently there are three widely used standardized patch tests: 1) Finn chamber, 2) True test and 3) Epiquick²⁸. In these tests, the suspected sensitizing agent is dissolved/suspended in a solvent (usually petrolatum). A patch containing the diluted agent is applied onto skin and read at 48, 72 and 96 hours. A patch test is interpreted based on observation of redness, itching and induration of skin at the site of the patch^{28,29}.

1.2.5 Biomonitoring for TDI Exposure

Biomonitoring of dNCOs involves either the measurement of specific antibody or of dNCO-conjugated biomolecules in blood, tissue or urine samples. Biomonitoring assays estimate total TDI exposure by converting TDI and its urinary metabolites to toluene diamine (TDA) by acid or base hydrolysis. A variety of analytical methods (e.g. chromatography) are used to determine the amount of TDA generated by laboratory hydrolysis^{30,31}. 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^{32,32,33}. This method does not distinguish between TDI and TDA exposure. Sabbioni *et al.*³⁴ reported a novel dNCO biomarker assay employing mild base hydrolysis of hemoglobin from methylene diphenyl diisocyanate (MDI) exposed rats to yield the hydantoin from the MDI conjugated lysine of the N-terminal valine^{34,35}.

Biological monitoring for TDI has been suggested and occasionally used to address

work place scenarios where individuals are involved in different work related processes with large variation of exposure levels. Sophisticated analytical methods are used to estimate, in urine or blood samples of TDI-exposed individuals, the total amount of the chemical exposure during a work shift. The advantage of this approach is that, in principle, the total dose for an individual may be estimated. However, due to inter-individual variation metabolism and pharmacokinetics, such biomonitoring data may greatly vary³⁶. It also must be noted that such biomonitoring methods only estimate an average exposure over an undetermined period, since for example in humans, the circulating half lives of hemoglobin and albumin are 120 and 15-20 days, respectively³⁵. MDI adducts to HSA have been reported from exposed workers¹⁶.

1.2.6 Diisocyanate Immunology and Asthma

Asthma is a clinical syndrome characterized by reversible airway obstruction, bronchial hyper-responsivity, and airway inflammation^{24,37}. Asthma represents a huge medical, social and economic burden, because its prevalence is increasing especially, in the developed world²⁴. Clinical similarities between diisocyanate-induced asthma and asthma caused by more common allergens, such as dust mite, suggest immunopathogenic mechanisms may be involved. OA is more difficult to identify and separate from general asthmas due to similarities in symptoms. Attempts, however to elucidate the pathogenesis of diisocyanate-induced asthma(s) are hampered by the high reactivity of these chemicals and the limitation of currently used animal models³⁸⁻

While asthma is considered an inflammatory disorder of the conducting airways, it is becoming increasingly apparent that the disease is heterogeneous with respect to immunopathology²⁴. TDI-specific IgE can be detected in only about 20% of the TDI-asthmatics, suggesting that other immunological pathways, other than Type I allergic mechanisms, may predominate in the majority of the asthmatics. Although the role of specific IgE antibody has been investigated, results thus far point to discrepancies or rather low associations between specific IgE antibodies and disease⁴¹⁻⁴⁵. Antibody detection against dNCO is difficult. This is normally done using a poorly characterized haptenated albumin. The contribution of inappropriate antigen in lack of specific-IgE detection in dNCO asthmatics is not known, but most studies evaluating different haptenated protein preparations, usually find differences in affinities of anti-TDI IgEs, but rarely identify a significant increase in TDI specific-IgE prevalence in TDI asthmatics.

The short circulating half-life of unbound serum IgE of about 2 days is of unique importance to occupational illnesses such as isocyanate asthma. Brief periods away from workplace may result in a decrease in serum IgE levels to levels undetected by conventional methods⁴⁶. Without accurate exposure information, negative isocyanate-specific IgE assays may lead to misdiagnosis and false conclusions about pathogenic mechanisms.

In addition, specific IgG antibodies have been found^{41,43,47-50} in sera of OA patients. TDI specific-IgG has been documented as a marker of exposure rather than

of disease⁵¹. The presence of dNCO specific-IgE and -IgG have been widely investigated as diagnostic markers of occupational asthma in diisocyanate-exposed workers^{13,40,40,41,46,50,52-55}. A presumptive diagnosis of dNCO asthma is made from work history, report of work-related asthma-like symptoms and nonspecific airway reactivity (to methacholine challenge). Presence of dNCO-specific IgE strengthens the diagnosis.

Conjugation (haptentation) 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. Diisocyanates have been shown to bind to skin and lung resident proteins. The major adducts found in the blood are to hemoglobin and albumin¹⁴. TDI-conjugated lung proteins have been co-localized with keratin, tubulin, laminin and actin¹⁴⁻¹⁷. It can be concluded from these reports, that, TDI binding, *in vivo*, demonstrates selectivity with respect to the target proteins. The ultimate antigenic protein(s), or all forms of chemical linkage(s) are however, not yet known.

A key aspect in the pathogenesis of dNCO asthma is the conjugation of dNCO with endogenous human airway proteins which include HSA, actin, and keratin. The antigenic epitopes resulting from the interaction of these conjugated proteins with the immune system and the subsequent steps leading to disease are poorly understood¹⁹. Figure 1.2 shows possible pathways in the immunopathogenesis of diisocyanate

asthma.

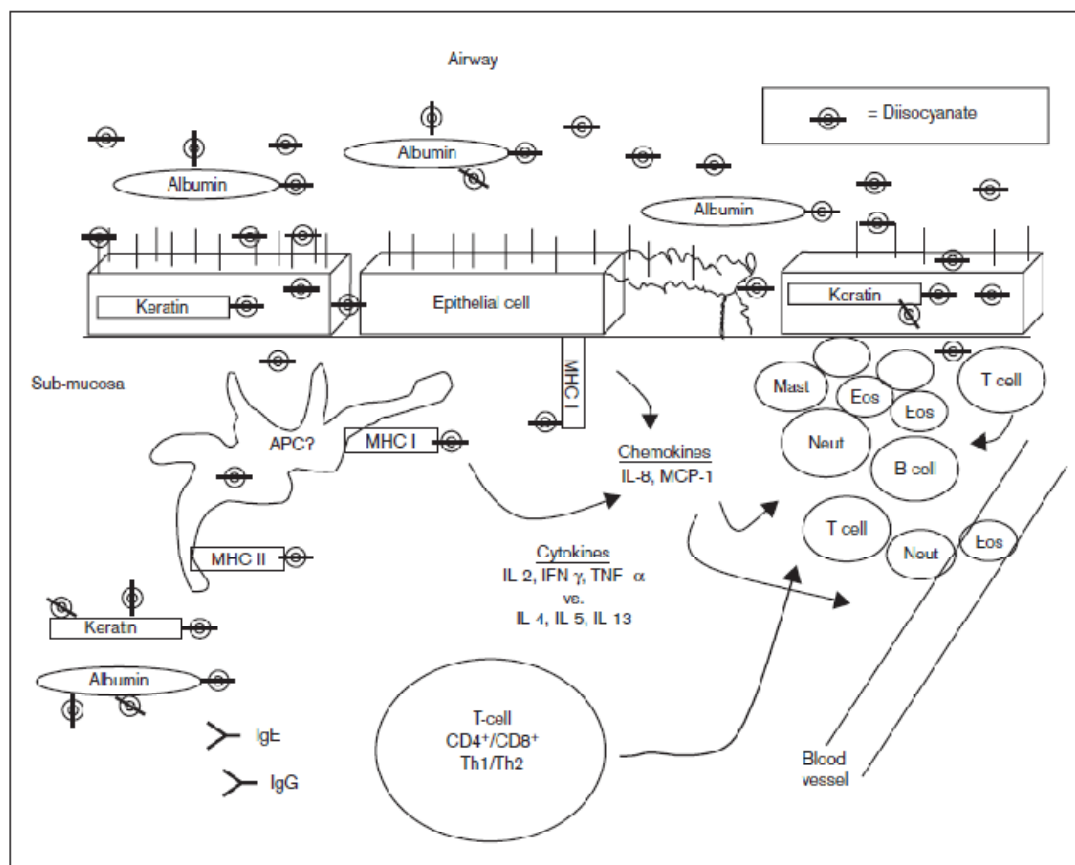


Figure 1.2: Hypothetical model for diisocyanate induced asthma¹³.

HDI has been shown to conjugate readily to human lung epithelial cell proteins after either vapor or liquid HDI exposure, providing evidence for the link between exposure and asthma^{51,56}. A mouse model of diisocyanate-induced asthma has been developed. Airway reactivity and pulmonary eosinophilic inflammation after inhaled TDI challenge are the hallmarks of this model.⁵⁷

Thiols such as glutathione and cysteine are prominent throughout the body. Under physiological conditions dNCOs bind preferentially, but reversibly, to thiols⁵⁸. This

reaction may be protective¹⁸ or may act as a carrier allowing the dNCO to be distributed within the body or into the cytoplasm.

The objectives of this thesis were:

A. Delineation of diisocyanate specific peptide/protein binding sites and chemical factors that favor protein hapteneration. The aim is to help understand the ultimate antigenic forms.

B. Production and characterization of relevant mAbs for use in biomarker/biomonitoring assays and as research tools from TDI vapor and TDI-conjugated proteins.

C. Isolation and identification of TDI conjugated proteins from TDI vapor-exposed cultured lung cells and from tissue of exposed mice. The mAbs identified from Part B, above, will be used to probe relevant TDI exposed tissue (both *in vitro* and *in vivo*) to identify the preferential protein/peptide targets for TDI conjugation.

CHAPTER 2

INSTRUMENTATION, MATERIALS AND METHODS

2.1 INSTRUMENTATION

2.1.1 Spectrophotometry

Protein determination and extent of conjugation using 2,4,6-trinitrobenzenesulphonic acid (TNBS) assay⁵⁹ were done on a Beckman DU800 (Fullerton, CA) ultraviolet/visible light (UV/Vis) spectrophotometer. The spectrophotometer was interfaced to an IBM PC computer and used the DU Series 800 Software (Fullerton, CA). Path length of the cuvette was 1 cm. A VWR Scientific 1140A (Niles, IL) circulating water bath was coupled to the Beckman DU800 for temperature regulation.

2.1.2 Mass Spectrometry (MS)

Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) MS techniques are able to detect minor mass changes in large biomolecules making them suitable for protein haptenation studies. Tandem mass spectrometry enables the acquisition and structural elucidation of protein or peptide sequencing. Both ESI and MALDI MS were employed in this project.

2.2.2.1 Electrospray Ionization

The ESI process can be summarized by three processes, which encompass droplet formation, droplet shrinkage and gaseous ion formation⁶⁰⁻⁶². A high potential was applied at the capillary tip (spray tip), while the analyte solution was passed through from a capillary at a constant flow rate. The mass spectrometer used in these studies was a Micromass QTOF-II™ (Waters Corporation, Milford, MA) quadrupole time-of-flight mass spectrometer (qTOF MS). Analyte ions were generated by positive-mode electrospray ionization (ESI). Samples were dissolved in 50/50 acetonitrile/water and pumped through a narrow, stainless steel capillary (75–150 μm internal diameter) at a flow rate of between 1 $\mu\text{L}/\text{min}$ and 1 mL/min .

A voltage of 3 or 4 kV was applied to the tip of the capillary, which was situated within the ionisation source of the mass spectrometer. The strong electric field, aided by a co-axially introduced nebulizing gas (nitrogen) flowing around the outside of the capillary, dispersed the solution emerging from the tip into an aerosol of highly charged droplets. The source block was maintained at 80 °C with the desolvation gas (nitrogen) maintained at 150 °C and a flow rate of 400 L/hr. The warm flowing nitrogen assisted in solvent evaporation and helped to direct the spray towards the mass spectrometer. The charged sample ions, free from solvent, passed through a sampling cone orifice into an intermediate vacuum region and from there through a small aperture into the analyzer of the mass spectrometer which was held under high vacuum. The lens voltages were optimised individually for each sample. The settings

varied with each set of experiments. Tandem mass spectrometry (MS/MS) data was generated by collision-induced dissociation (CID) with argon. Visualization and analysis of data was done using the Micromass MassLynx™ 4.0 software suite for Windows 2000 (Waters Corporation, Milford, MA).

2.1.2.2 Matrix Assisted Laser Desorption Ionization (MALDI)

The MALDI technique, first introduced in 1988 by Hillenkamp and Karas,⁶³ was used to analyze biomolecules. Samples were diluted with UV absorbing matrix solution (50/50 acetonitrile/2% trifluoroacetic acid) and deposited as 1 µL aliquots of each sample on a gold sample chip (Ciphergen Biosystems, Inc., Fremont, CA) and allowed to air-dry. The MALDI instrument was a linear TOF mass spectrometer (PBS IIc, Ciphergen Biosystems, Inc.). All spectra were acquired using a laser power at or slightly above the threshold for ion production (laser step 140-160 nm). A total of 130 individual spectra (single laser shots) were averaged into a composite spectrum for each sample spot. When laser beam irradiated analyte-matrix mixture on the chip, indirect vaporization of the analyte resulted when the strongly absorbing matrix vaporized and carried the analyte with it. Ionization resulted from the proton donation of the matrix to the analyte (*positive mode*). Individual spectra were calibrated, baseline corrected, and normalized to total ion current (TIC), followed by identification of peak “clusters” representing peaks common to groups of spectra. Data

analysis were performed using Biomarker Wizard™ software (CIPHERGEN Biosystems, Inc., Fremont, CA).

The Micromass Maximum Entropy™ (MaxEnt, Waters Corporation, Milford, MA) algorithm was utilized in these studies. The algorithm derives from the operating principle which maximizes the probability of getting the parent mass o given an apparent mass spectrum (m/z) while assuming Gaussian (normal) statistics for the noise distribution⁶⁴. Post acquisition mass spectral charge deconvolution was performed with the “MaxEnt3” algorithm in the ProteinLynx™ 4.1 software suite (Waters Corp., Milford, MA).

2.2. Materials and Methods

When planning the production of monoclonal antibodies production considerations must be given to the sensitizing and screening hapten/antigen(s) including method of antigen preparation, immunization procedure, and the choice of animal to immunize.

2.2.1 Conjugation of Diisocyanates, Monoisocyanates or Diisothiocyanates to Proteins

Keyhole limpet hemocyanin (KLH, hemocyanin from *Megathura crenulata*), mouse serum albumin (MSA, fraction V), human serum albumin (HSA, fraction V), lysozyme (chicken egg white), keratin protein (derived from hair, wool, horn, nails or other similar tissues in animals), and collagen (calf skin type 1 species) were obtained from Sigma Aldrich (St. Louis, MO). Dimethyl phenyl isocyanates (DMPI) (2,3 DMPI, 3,5 DMPI and 2,5 DMPI) were obtained from Alfa Aesar (Wade Hill MA). 2,4 toluene diisocyanate (2,4 TDI) , 2,6 toluene diisocyanate (2,6 TDI), hexamethylene diisocyanate (HDI), o-toluene isocyanate (OTI), p-toluene isocyanate (PTI), phenyl isocyanate (PI), toluene diisothiocyanate phenyl isocyanates (2,4 TITC and 2,6 TITC) and methylene bis-cyclohexylisocyanate (MDI) were obtained from Sigma Aldrich (St. Louis, MO). These proteins, without further purification, were used for mouse inoculation, mAb production, and ELISA sandwich assays.

Diisocyanates, Monoisocyanates and Diisothiocyanates Conjugated to Proteins		
Abbreviation	Name	Structure
2,4 TDI	2,4 Toluene Diisocyanate	
2,6 TDI	2,6 Toluene Diisocyanate	
MDI	4,4 Methylene Diphenyl Diisocyanate	
HDI	Hexamethylene Diisocyanate	
2,5 DMPI	2,5 Dimethylphenylisocyanate	
2,3 DMPI	2,3 Dimethylphenylisocyanate	
3,4 DMPI	3,4 Dimethylphenylisocyanate	
PTI	4 Toluene Isocyanate	
OTI	2 Toluene Isocyanate	
PI	Phenyl Isocyanate	
2,4 TITC	2,4 Toluene Diisothiocyanate	

Figure 2.1: Chemical structures for some of the isocyanates conjugated to carrier proteins.

All proteins were prepared at 5 mg/mL in phosphate buffered saline (PBS), pH 7.4. Five μ L aliquots of each dNCO, mono-isocyanate (NCO) or dithioisocyanate (dNCS) were added to 450 μ L dry HPLC grade acetone (Sigma, MO) and infused with rapid stirring into the protein solution at a rate of 1.2 mL/hr at room temperature (RT) using a syringe pump (model 100; KD Scientific Inc., Holliston, MA, USA), until a molar ratio of 1:40 protein:dNCO (NCO or dNCS) was achieved. The resulting conjugates were centrifuged at 300XG for 10 minutes then dialyzed against 3X buffer changes in 1X PBS at 4°C using molecular porous membrane tubing obtained from Spectrum Laboratories, (Rancho Dominguez, CA MWCO:12-14 000 kDa). HSA was acylated with acetic anhydride to block all available primary amines prior to reaction with 2,4 TDI in a separate preparation. The conjugates were filtered through 0.45 μ m syringe filters (Millipore, Billerica, MA, USA) and stored in aliquots at -20 °C until use.

2.2.2 Protein Determination

Protein determinations were performed on the conjugates using the Bradford method⁶⁵ with HSA as standard. The standards were in the range 5-100 μ g/mL protein of HSA and absorbance measured at 595 nm on a spectrophotometer. Fifty μ g/mL of conjugated protein and 50 μ g/mL carrier protein were assayed similarly to standards.

2.2.3 TNBS Assay

The TNBS reagent primarily assesses chemical adduction with primary amines of amino acids⁵⁹ on the surface of the protein.

Stock TNBS was made at 5% (50 g/L) and a borate buffer of 0.1 M pH 9.3 (8.796 g/L). The TNBS was diluted at 1:5.68 TNBS:borate buffer with the borate buffer. 25 μ L of TNBS were added to 1 mL of sample protein diluted in $\frac{1}{2}$ saturated borate buffer and allowed to react for 30 min and read at 420 nm. Albumin standards included 5, 25, 50, 250 and 500 μ g/mL. A protein sample dilution with a resulting concentration of 50 μ g/mL was made and read at 420 nm on a Beckman DU800 spectrophotometer (Fullerton, CA).

2.2.4 Preparation of Isocyanate-Peptide Adducts

All chemical were from Sigma Aldrich. Formic acid (99.5%) was of mass spectrometry grade, and acetonitrile and acetone were high performance liquid chromatographic (HPLC)-grade. Ortho-toluene isocyanate (OTI), para-toluene isocyanate (PTI), 2,4 toluene diisocyanate (2,4 TDI) and 2,6 toluene diisocyanate (2,6 TDI) were dissolved in HPLC-grade acetone. The peptides Leu-enkephalin (Leu-enk, YGGFL), Angiotensin I (DRVYIHPFHL), Substance P-amide (RPKPQQFFGLM-NH₂), and Fibronectin-adhesion promoting peptide (FAPP, WQPPRARI) were dissolved in 18 m Ω distilled deionized water (DDI) produced by a Millipore Synthesis A-10 (Billerica, MA). Prior to mass spectrometry analysis, peptide-NCO conjugates were diluted in 50/50 0.1% formic acid in DDI water/HPLC-grade acetonitrile.

Peptides were dissolved in DDI at a concentration of 0.5 mg/mL. Equimolar solutions of each of the four isocyanates were prepared in 1 mL HPLC-grade acetone and infused into each peptide solution at a rate of 1.2 mL/hr using a syringe pump (Model 100, KD Scientific Inc., Holliston, MA) with constant stirring. Reactions were carried out at room temperature in a chemical fume hood and allowed to stir for 15 minutes following completion of the infusion. Peptide-isocyanate adduct solutions were filtered using 0.45 μ m filters (Millipore, Billerica, MA) and stored at -20°C until analysis.

2.2.5 Mass Spectrometry for Peptide Reactivity

Each peptide adduct solution was diluted 50:1 in 50/50 0.1% formic acid/HPLC-grade acetonitrile, yielding a final solution concentration of all peptide-NCO species of approximately 100 pmol/ μ L. Samples were infused at a rate of 5 μ L/min to a high performance liquid chromatographic-quadrupole-time-of-flight micromass (HPLC- Q-TOF2) (Waters Corp., Milford, MA) mass spectrometer operated in positive electrospray (+ESI) mode. Sample analysis was performed at a capillary voltage of 2.5 kV with dry N₂ desolvation gas (NitroFlowLab, Parker Hannifin Corp., Haverhill, MA) at a flow rate of 400 L/hr and a temperature of 150° C. Ultra High Purity (UHP) Argon was used as a collision gas at collision energies of 5 eV (MS analysis) and 25-30 eV (MS/MS analysis). For peptide sequencing, the doubly charged [M+2H]²⁺ ion was selected by MS¹, with the exception of Leu-enkephalin (the [M+H]⁺ ion was selected). The collision energy was set independently for each peptide to yield optimal

relative abundance of sequence-specific fragment ions. Post-acquisition mass spectral charge deconvolution was performed with the “MaxEnt3” algorithm in the ProteinLynx™ 4.1 software suite (Waters Corp., Milford, MA). The mass spectrometer was externally calibrated over the range 70-1570 atomic mass units using the CID fragment ion mass spectrum of [Glu]¹-fibrinopeptide B (EGVNDNEEGFFSAR) [M+2H]²⁺ ions acquired at collision energy of 35 eV.

2.2.6 Experimental Animals

Female specific-pathogen-free inbred C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 5 to 6 weeks of age, and used for vapor exposure while BALB/c mice were used for the rest of the antibody production. Upon arrival, the mice were quarantined for 2 weeks and acclimated to a 12-hour light/dark cycle. Animals were housed in ventilated microisolator cages under environmentally controlled conditions at the National Institute for Occupational Safety and Health (NIOSH) animal facility in compliance with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved guidelines and an approved Institutional Animal Care and Use Committee (IACUC) protocol. The animal rooms were monitored for specific pathogens through a disease surveillance and sentinel animal program. Food and water were provided *ad libitum*.

2.2.7 TDI Exposure System

Toluene diisocyanate (TDI; Mondur TD 80 Grade A; 80/20 mixture of 2,4 and 2,6 isomers, respectively) was provided by Bayer Corporation, Polyurethanes Division (Pittsburgh, PA). The TDI exposure system has been described in detail previously^{39,66}. Briefly, mice were exposed in a 1200 L stainless steel live-in chamber (Unifab Corporation, Kalamazoo, MI) supplied with high efficiency particulate air (HEPA) purified and conditioned air supply providing nine air changes per hour and maintaining temperature and humidity at 23 ± 2 °C and 50 ± 5 %, respectively. Mice were housed individually in hanging stainless steel mesh cages and remained in the chamber on weekdays and were returned to ventilated shoe box style cages on weekends. Generation of a TDI vapor atmosphere was achieved by passing dried HEPA filtered air over a 50 cm² surface of liquid TDI followed by dilution to the desired concentration with HEPA filtered and humidified air. The TDI concentration (50 ± 5 ppb) in the chamber was continuously monitored using a Remote Intelligent Sensor (RIS) TDI analyzer (Scott Safety and Health, Monroe, NC). The RIS units were calibrated against a fluorecamine assay with a detection limit of 10 ng/mL as previously described⁹. Five mice were exposed to TDI vapor for 4 hr/day, for 12 consecutive workdays. Lymph nodes and spleens were collected 24 hrs following the final exposure.

2.2.8 2,4 and 2,6 TDI Immunization Regimen

BALB/c were immunized 4-6 times, intraperitoneally, at biweekly intervals. Mice were primed with 50 µg of 2,4/2,6 TDI-KLH and that antigen concentration maintained for each subsequent booster immunization. Mice were immunized with 2,4/2,6 TDI-KLH at the 1:40 (KLH: dNCO) molar ratio emulsified in TiterMax®. The tail vein bleeds were taken before antigen boosting every other week and compared with the baseline bleeds to determine the development of specific immunological sensitization. The sera (polyclonal) antibodies were screened for specificity to 2,4/2,6 TDI-HSA and also investigated to see if they can discriminate against other forms of isocyanate-protein conjugates to validate that the antibodies were protein carrier-independent. This was done using the conjugates as solid-phase antigen in an alkaline-phosphatase-based indirect ELISA. A final boost of 50 µg antigen without the adjuvant was given three days prior to hybridoma production.

2.2.9 Monoclonal Antibody Production and Storage

Hybridomas were produced following standard techniques as previously described⁶⁷ using SP2/0-AG14 myelomas (American Type Culture Collection (ATCC) # CRL-1581) as fusion partners and polyethylene glycol (PEG) (molecular weight 1500 Da) as the fusagen.

Mice were euthanized by CO₂ asphyxiation. Spleens and (lymph nodes in the case of TDI vapour exposed mice) were removed using aseptic techniques. All work was carried out under sterile conditions in tissue culture room. All surfaces were wiped

with 70% alcohol prior to use and work was carried out in Sterigurd III Advance® class II biological safety cabinet (The Baker Company, Sanford, ME).

A spleen cell suspension was formed using a scalpel to gently free the cells from the capsule into a petri dish containing 10 mL of serum-free Dulbecco's Modified Eagles Media (DMEM) (Life Technologies, Rockville, MD). The spleen cell suspension, free from large particulates, was transferred to a 15 mL centrifuge tube and centrifuged at 300 G for 5 min at 4°C. The cells were resuspended in 1 mL of pure water for red cell lysis for 1 minute and three rinses in 10 mL of serum-free DMEM were performed to remove burst red blood cells. The final step was to suspend the spleen cells in 4 mL of fetal bovine serum (FBS) .

Mouse spleen cells (1×10^8 to 1×10^{15}) were fused in a 10:1 ratio with SP2/0-AG14 myeloma tumor cells PEG solution. The fused cell suspension was diluted in 100 mL of complete DMEM supplemented with 1 mM pyruvate, 100 units/mL penicillin, 100 µg/mL streptomycin, 0.292 mg/mL L-glutamine, 100 µM sodium hypoxanthine, 16 µM thymidine, 10% fetal calf serum (HyClone, Logan, UT) and 100 units/mL IL-6 (Boehringer Mannheim, Germany) containing hypoxanthine, aminopterin, and thymidine (HAT) (Sigma, MO).

Cell cultures were incubated at 37°C with 10% CO₂ (model 370 Steri-Cycle CO₂ incubator, Forma Scientific Inc., Marietta, OH). After 1 week, the wells were scored

for clone growth using a Leitz-Wetlar inverted light microscope. One hundred μL of complete media per well was replaced with hypoxanthine , aminopterin and thymidine (HAT) selection media plus colony stimulating factor IL-6 and cells were allowed to grow for another week. Two weeks post fusion, HAT cell culture media was replaced (100 μL /well) with complete DMEM plus IL-6. Hybridoma containing wells were screened after sufficient growth. Indirect ELISA was used to determine antibody secreting hybridomas to the selected protein. Positive wells on the screening plate indicated antibody secreting hybridoma wells on the cell fusion culture plate. Prior to freezing and limiting dilution, antibody secreting hybridoma cells (100 μL cell suspension) were transferred to 24-well tissue culture plates (Nalge Nunc) for expansion in complete DMEM plus IL-6 (2 mL/well). After sufficient growth, cells were screened against their respective antigen for a second time to confirm antibody secreting hybridomas. Positive hybridomas were cloned by limiting dilution. Hybridomas (100 mL cell suspension) were seeded onto a 96- well tissue culture plate in complete DMEM plus IL-6 media and serially (1:2) diluted across the plate to isolate a single hybridoma and establish true individual clones. During the fusion process, multiple hybridomas could grow within the same well, so limiting dilution was necessary to establish an individual hybridoma and not a collection of hybridomas. In addition to clonal limiting dilution, a 1 mL cell suspension from the 24- well plate was transferred to a tissue culture flask in complete DMEM media for further expansion and freezing to ensure hybridoma preservation. Single colonies on the dilution plate were identified and screened by indirect ELISA to confirm antigen

specific antibody secreting hybridomas and the best three colonies per plate were selected for expansion, freezing, antibody production, and subsequent use. Selected hybridomas were then grown in tissue culture flasks in complete DMEM media to collect sufficient quantities (200 to 300 mL) of supernatant fluid for use. After complete cell coverage of the flask, 50 mL of media was added to the flask and 3 to 5 days later, the media was collected. The process was repeated until sufficient (200 to 300 mL of cell culture supernatant fluid) quantities of mAbs were harvested. Aliquots of stable TDI-specific hybridomas were frozen in a mixture of 10% (v/v) dimethylsulfoxide and 90% fetal calf serum for storage in liquid nitrogen.

2.2.10 Enzyme-linked Immunosorbent Assay (ELISA) Formats

Format for hybridoma screening.

The screening assay was a critical stage in antibody production because the reagents generated are only as good as the selection system employed. The immune system generates a wide range of specificities and affinities. It was important that mAbs were screened against HSA -TDI conjugates and appropriate controls like KLH and HSA included so that only antibodies with desired specificity were selected or otherwise valuable time would be lost due to false positives or false negatives.

Hybridoma screening tests were carried out using an indirect ELISA according to Schmechel *et.al*^{68,69}. MaxiSorp™ ELISA plate wells (Nalge Nunc International, Naperville, IL) were coated with 5 µg/mL of 2,4 TDI -HSA and incubated overnight

at room temperature (RT). The plates were kept in a plastic box containing moist filter paper. Following overnight incubation and all subsequent ELISA steps, wells were washed 3 times with 200 μ L PBS with 0.05 % Tween 20 (PBST) /well. The plates were blocked by incubating for 1 hr at RT in 200 μ L/well PBST containing 1% non-fat dry milk powder (PBSTM). Initial hybridoma culture supernatant (CSN) fluids were diluted 1/5 in PBSTM and 100 μ L/well were incubated for 1 hr at 37 °C. Bound antibodies were labeled by incubation with 100 μ L/well of Biotin-SP-conjugated Affinity Pure goat anti-mouse IgG+ IgM secondary antibody diluted 1/5000 in PBSTM (Jackson Immuno Research Laboratories, Inc., West Grove, PA) for 1 hr at 37° C. One hundred μ L of alkaline phosphatase-conjugated streptavidin at a dilution of 1/5000 in PBSTM (Jackson Immuno Research Laboratories, Inc., West Grove, PA) was added and incubated for 1 hr at 37 °C. The ELISA was developed by incubating 100 μ L p-nitrophenyl phosphate-containing 2 mg /mL in (1M diethanolamine, 0.001M MgCl_2 in distilled water, pH 9.8) at RT for 30 minutes. The optical density (OD) was determined spectrophotometrically at 405 nm using an UltraMicroplate Reader, Model ELx800 (BIO-TEK Instruments, Inc., Winooski, VT). Assay background controls were processed in parallel and contained plain cell-free culture medium instead of mAb supernatant fluid. Optical densities greater than 3 times the OD at 405 nm of the background controls were considered to be positive.

Antibody Isotyping and Quantification.

Antibodies produced by TDI-specific hybridomas 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 e.g 2,4 TDI-HSA, blocked and washed as described for the screening ELISA. To determine the isotype, bound mAbs were incubated with 100 μ L of Biotin-SP-conjugated goat anti-mouse isotype-specific secondary antibodies (IgG₁, IgG_{2a}, IgG_{2b}, or IgG₃).

The monoclonal antibodies were quantified using isotype specific ELISA kits (Jackson Immuno Research Laboratories, Inc., West Grove, PA) according to the manufacturers' instructions. The amount of specific antibody in the supernatant fluid was determined from a standard curve generated with specific antibodies of known concentrations. The standards and supernatant fluid were assayed in parallel.

Antibody and antigen titration.

Antibody and antigen titrations were done to determine the optimum concentration of antibody and coating antigen to use for the reactivity studies. Plates were coated with varying amounts of 2,4-/2,6 TDI-HSA from 40 μ g/mL to 0.125 μ g/mL, overnight, and the solid phase antigens were reacted to antibodies serially diluted from 1:2000 to 1:564000. A range of antigen concentration and antibody concentration within the linear range was chosen for reactivity assays.

ELISA Format for mAb reactivity.

The mAb's reactivity toward proteins conjugated to several different dNCOs, NCOs and dNCSs was tested using an alkaline phosphatase-mediated indirect ELISA.

PolySorp™ ELISA plate wells (Nalge Nunc International, Naperville, IL) were coated with 5 µg/mL of test antigen by overnight incubation at RT. The plates were processed as previously described for the antibody screening ELISA except Biotin-SP-conjugated Affinity Pure goat anti-mouse IgM or IgG at a dilution of 1/5000 was used as secondary antibody. The results represent the average OD_{405nm} of 4 ELISA well replicates, which were corrected by subtracting the average OD_{405nm} of 4 ELISA background control wells.

ELISA format for affinity constant (K_a) measurements.

The method described below is derived and adopted from Beatty⁷⁰ *et.al*.

Standardization ELISAs were carried out to determine the antibody and antigen concentration that would result in saturation kinetics. For 2,4 TDI-HSA antibodies the antigen concentrations used were 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL and 0.312 µg/mL. For 2,6 TDI-HSA antibodies the antigen concentrations used were 0.625 µg/mL, 0.3125 µg/mL, 0.156 µg/mL and 0.0781 µg/mL and antibody concentrations ranged from 10^{-7} to 10^{-10} M in all the assays. The rest of the steps were identical to screening ELISA.

In summary, ELISA were plates pre-coated with four different concentrations of 2,4-/2,6 TDI-HSA ($[Ag]^i$, $[Ag]^{ii}$ to $[Ag]^{iv}$) and were separately incubated with serial concentrations of each mAb. Sigmoid curves were constructed using the OD values obtained for different concentrations of each mAb to calculate the affinity constant. The half maximum OD (OD-50) was determined for all selected curves from which the corresponding antibody concentration ($[Ab]^i$, $[Ab]^{ii}$, to $[Ab]^{iv}$) was extrapolated. Accordingly, $[Ab]^i$ and $[Ab]^{ii}$ are the measurable total Ab concentrations at ODⁱ-50 and ODⁱⁱ-50 for plates coated with $[Ag]^i$ and $[Ag]^{ii}$, respectively. The affinity constant was finally determined using the following equations:

$$Ab + Ag \rightleftharpoons AbAg \quad (1)$$

$$\text{Then, } K_a = \frac{[AbAg]}{[Ab][Ag]} \quad (2)$$

$$\text{For } [Ag^{ii}] = [Ag^i] / 2 \quad (3),$$

$$K_a = (n - 1) / 2(n[Ab^{ii}] - [Ab^i]) \quad (4)$$

Where Ab = antibody, Ag = antigen, AbAg = antibody-antigen complex and $n = [Ag]^i / [Ag]^{ii}$ resulting in 6 calculations of K_a possible with 4 different antigen concentration⁷⁰. Curves were generated by using a four parameter logistic model (Hill-Slope model) and used to calculate OD-50.

2.2.11 Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS–PAGE).

Gel electrophoresis was performed using 10% SDS–PAGE gel and run in tris/glycine/SDS buffer (Bio-Rad, Hercules, CA) at room temperature on a Mini-PROTEAN II® electrophoresis unit (Bio-Rad, Hercules, CA) set at 100 volts for 2 hrs. A sample aliquot of the protein conjugate was combined with equal volumes (1:1) of Laemmle sample buffer, 62.5 mM Tris-HCl, pH 6.8; 2.0% SDS (w/v); 25% glycerol; 2.5% β -mercaptoethanol ; and 0.01% (w/v) bromphenol blue and then heated (90 to 100 °C for 5 to 10 min) to denature and reduce the proteins in the presence of SDS. Gel lanes were loaded with equal amounts of total protein or equal volumes. After completing the separation, the gel was placed in a plastic container, rinsed with distilled deionized water, stained using GelCode® staining reagent (Pierce, Rockford, IL) for 1 h, then destained using distilled deionized water for band visualization or used in Western blotting.

2.2.12 Western Immuno-blotting

After SDS-PAGE protein separation, the gel was placed in a dish and rinsed with distilled deionized water. The gel was then loaded into a Mini Transblot Cell (Bio-Rad) and the protein bands transferred overnight (4 °C) in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.05% (w/v) SDS) to a nitrocellulose membranes (0.2 μ m, Bio-Rad) using a setting of 40 mA. To prevent non-specific binding, the membrane was placed in a plastic container with 50 mL of 3% bovine

serum albumin and incubated for 2 h at room temperature on an orbital shaker. After blocking, the membrane was rinsed three times with 50 mL of PBST. The membrane was incubated for 1 hr with a 1/10 dilution of mAb culture supernatant for 1 h. After washing, the blot was incubated with Biotin-SP-conjugated Affinity Pure goat anti-mouse IgM/IgG secondary antibody at a dilution of 1/5000 in 3% BSA in PBST for 1 h at 37 °C. Immune complexes were labeled with alkaline phosphatase-conjugated streptavidin by incubating a 1/5000 dilution in 3% BSA in PBST for 1 hr at 37 °C. Protein bands were visualized using a nitroblue tetrazolium/bromo-chloro-indolyl phosphate substrate reagent kit (NBT/BCIP Promega, Madison, WI). Color was allowed to develop for 5 min and stopped by washing the membranes with distilled/deionized water.

2.2.13 ELISA Dot Blot

A dot-blot analysis was carried out to evaluate the mAb reactivity towards native and denatured proteins and conjugates. Native TDI-conjugates (4 µL of 5 µg/mL of antigen per spot) were spotted onto a nitrocellulose membrane (0.2µm, Bio-Rad) and allowed to dry overnight. The dot blots were repeated using denatured carrier proteins and TDI conjugates that, were treated with (β-)2-mercaptoethanol (final concentration 25 mL/L) at 100 °C for 10 minutes prior to spotting. All the other steps were identical to the Western blot protocol with regard to incubation times and color development.

2.2.14 *In vitro* Exposure System for Cells and Protein Solution

Mouse serum albumin (MSA, 2 mg/mL, Sigma) or live A549 and BEAS-2B cells (ATCC # CCL-185 and CRL-9609 respectively) were exposed to vapor phase TDI using a VITROCELL[®] exposure system (VITROCELL Systems, Inc. Waldkirch, Germany). A549 cell line was initiated in 1972 by Giard *et al*⁷¹ through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. BEAS-2B cell line are epithelial cells isolated from normal human bronchial epithelium obtained from autopsy of non-cancerous individuals⁷². Cells were seeded on Transwell[®] culture inserts and grown at 37 °C/5% CO₂ to confluence prior to exposure. A vapor atmosphere of 50 ppb TDI was generated using the animal exposure system at NIOSH according to Johnson *et al*³⁹. 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 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. Air flow rate (static open air, 20, 100 and 200 mL/min) and exposure time (1, 2 and 4 hrs) were varied to determine their impact on protein conjugation. Flow rate of 100 mL/min approximates the minute ventilation of a mouse. The exposure system was maintained at 37 °C throughout the entire exposure using a circulating water bath. Immediately following exposure, MSA solution was aliquoted and frozen at -80 °C until analysis. Cells were harvested using 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.

Western immuno-blot detection of TDI-protein conjugation

One µg of TDI-MSA conjugate (positive control, prepared in solution as per Johnson *et. al*³⁹ 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 one hour 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 mAbs (Clones 60G2, 2E5, 62G5, 77E6, 79G7, 67C4, 66F7) 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 1h with a 1:15,000 dilution. The proteins were detected using an Odyssey infrared imaging system (Li-Cor Bioscience Lincoln NE).

CHAPTER 3

CHARACTERIZATION OF ISOCYANATE SPECIFIC BINDING SITES ON PROTEIN-ISOCYANATE CONJUGATES.

3.1 Introduction

It is imperative to understand better the bioorganic and physical chemical reactions of dNCOs under biologically-relevant conditions. Incorporation of this understanding in the production of tools with applications in both biological and dNCO disease mechanistic investigations is of paramount importance. Reactive low molecular weight (LMW) chemicals are incomplete antigens and as such are incapable of eliciting specific IgE or IgG antibody responses alone⁷³. A LMW chemical may act as a hapten to form complete antigen by combining with autologous host proteins present in the respiratory tract or skin. This chapter presents the preparation and characterization of antigens that were used for the production and subsequent analysis of monoclonal antibodies. Reagent grade chemicals with a high degree of purity were used for the preparation of conjugates.

Chemical characterization was used to determine whether conjugation had been achieved and to semi-quantitate the ratio of chemical ligands bound per molecule of carrier protein. Chemical characterization of TDI-haptenated proteins is a very difficult task, as TDI can polymerize, and form inter/intra cross-links with protein sites. Several techniques were employed to evaluate the TDI conjugated proteins.

These included:

(1) loss of primary amines from the protein as assessed by loss of reactivity to TNBS⁷⁴⁻⁷⁶. The isocyanate binding fraction was calculated as the percent of amine groups that had reacted with isocyanates according to Lemus *et al*⁵⁵ as follows:

$$\%S = 100 - \frac{[100 \times A^*]}{A} \quad (3.1) \quad \text{where } S \text{ is the substitution, } A^* \text{ is the absorbance}$$

at 420 nm of the isocyanate conjugate and A is the absorbance 420 nm of the carrier protein. The number of carrier groups was determined before and after conjugation and the difference in amino acids is the number of hapten groups bound to carrier amines.

(2) shift in average protein mass as assessed by MALDI-TOF-MS and gel electrophoresis, and

(3) identification of specific binding sites following protein enzymatic digestion and analysis by HPLC-Q-TOF.

The basis of allergen serology is the specific three-dimensional structure of the allergen⁷⁷, which remains incompletely defined for dNCO asthma and has been a major obstacle to research and clinical studies. Isocyanates change protein structure upon reaction creating neo-epitopes^{13,19} and are thus more challenging to standardize compared to other allergens. A theoretical understanding and technical methods for generating biologically relevant isocyanate antigens is crucial to diisocyanate immunoassays and hence, extensive characterization of the antigens, by methods outlined above. Wisnewski and colleagues⁵¹ noted standardization issues regarding

various methodologies particularly in reference to conjugate preparation. Although it would be desirable to generate dNCO-protein conjugates under physiologic or real life human exposure conditions, this is difficult to achieve experimentally, in part due to practical reasons and that the exact immunologic proteins/epitopes are not fully understood. Conjugate preparation is a multi-step process that has to take into account the following variables:

- (1) The particular diisocyanate or commercial product to be conjugated
- (2) The protein carrier
- (3) Reaction conditions (e.g., the protein concentration, mixing strategies, reaction time and temperature), and
- (4) Post reaction-processing (i.e., stopping reaction , conjugate isolation and purification)

3.2 Results and Discussion

3.2.1 Choice of Isocyanates

In theory, it makes sense to prepare the conjugates using commercially relevant starting material (e.g., polymeric MDI, HDI prepolymer or 80/20% TDI). In the initial study commercially produced 80/20% TDI mixture was used to expose mice to 50 ppb vapor. However, optimal vapor exposure conditions for mAb production are not known and hyperimmunization may be desired to achieve mAbs with a range of TDI-protein selectivities. Thus, conjugates prepared using the monomeric forms of TDI,

MDI or any other isocyanates were used for both sensitization and mAb characterization. The TDI-adduct formation was modified by (1) changing position of NCO group on benzene ring, (2) removing one NCO, (3) substituting one NCO with a methyl group, or (4) substituting dNCO groups with dNCS groups (See Figure 2.1).

The isocyanate functional groups in TDI can potentially react with a hydroxyl group (in hydrophobic pockets) to form a urethane linkage, a thiol group to form a thiourea, or an amine group to form a urea. 2, 4-TDI is an asymmetrical molecule and thus has two isocyanate groups of different reactivity. The 4-position is more reactive than the 2-position because it is more accessible²¹. 2, 6-TDI is a symmetrical molecule and thus has two isocyanate groups of similar reactivity, similar to the 2-position of 2, 4-TDI. Reaction of one isocyanate group will cause a change in the reactivity of the second isocyanate group because both isocyanate groups are attached to the same aromatic ring. The first NCO of a diisocyanate may form a urea linkage with a primary amine of a protein while the second NCO may be hydrolyzed to an amine with the potential to react with additional NCO groups to undergo intra- or intermolecular cross linking with other proteins resulting in dimers, trimers and so forth¹². Intra- and intermolecular crossing can also be mediated by a single dNCO molecule reacting to 2 separate amino acids.

3.2.2 Choice of Carrier Protein

In vivo conjugation of TDI to proteins in the human airways is thought to be a primary event in TDI exposure⁴⁰. However, the characterization of dNCO haptenated proteins is complicated by the ability of the dNCOs to haptenate multiple proteins, to self-polymerize and form both intra- and intermolecular cross-links with diverse proteins and non-protein species.

HSA is the protein most widely used in the preparation of LMW chemical-protein conjugates. Protein carriers other than HSA have also been used successfully for this purpose, although they have been of limited clinical use with such antigens⁷⁸. It is important that the right conjugation ratio is achieved since the immunologic reactivity of the resulting conjugate may be different depending on the number of exposed antigenic epitopes⁷⁹.

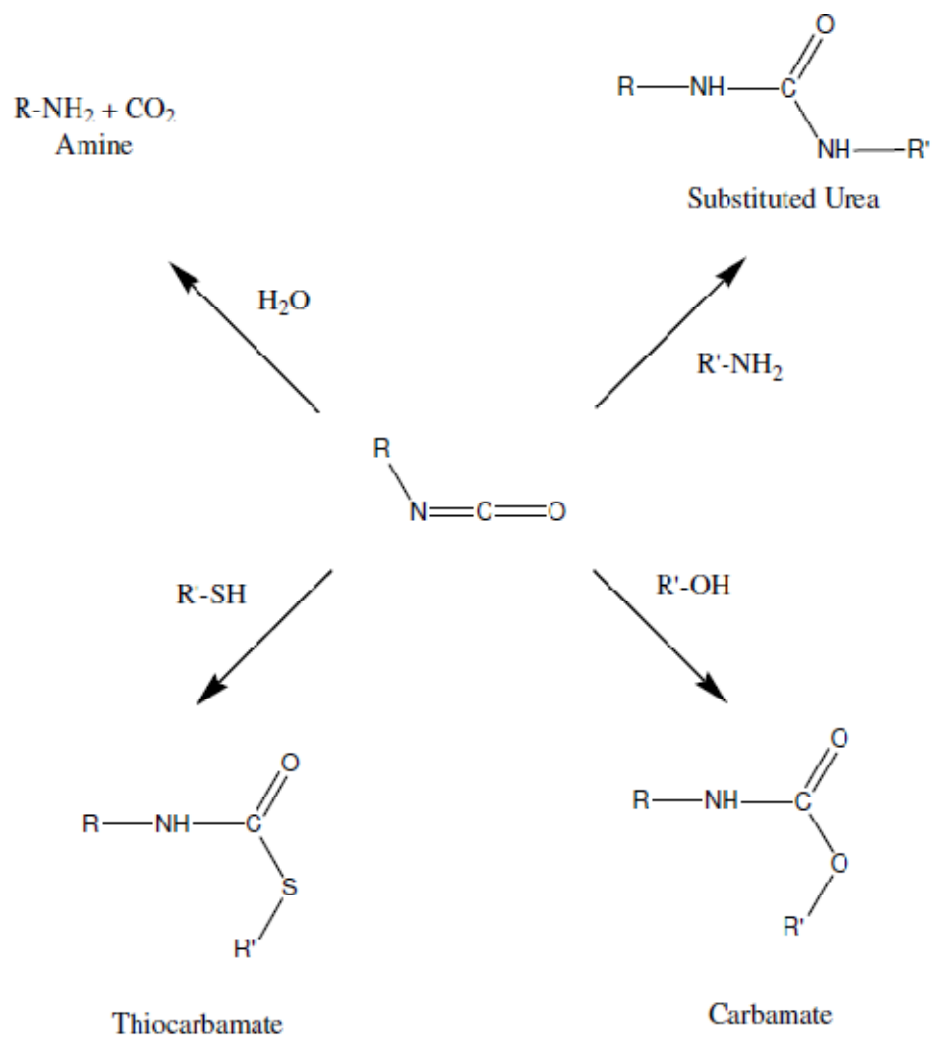
The antigens used in the characterization of the mAbs produced in this thesis were divided according to (a) the chemical nature of the carrier protein as basic (lysozyme), acidic (HSA) or insoluble (keratin); (b) the origin of the carrier protein as animal or human (MSA or HSA).

Diisocyanates may react with a number of different proteins and peptides present in the airway fluids and tissues. In most studies, HSA has been the carrier of choice in preparing diisocyanate conjugates. Wisnewski *et. al*¹⁵ found HSA to be the

predominant soluble extracellular HDI-conjugated protein in airway lavage fluid of subjects exposed to an HDI aerosol, whereas keratin 18 was the predominant diisocyanate conjugate in endo bronchial biopsy samples. It has also been shown that serum albumin is the major protein in plasma to which both MDI and TDI adducts are formed^{16,80}. Studies have also identified other molecules in the respiratory tract that are modified by isocyanates including laminin, tubulin¹⁷ and glutathione^{81,82}. HDI-HSA conjugates have been shown to elicit human innate system responses⁸³. In this thesis, HSA was chosen as our carrier protein for the screening assay of monoclonal antibodies. Other proteins chosen for the characterization of the antibodies, these included keratin, lysozyme and MSA. Evaluation of cross-reactivity was also carried out with analogous mono- and di- isocyanates using either standard or inhibition assays.

3.2.3 Reaction Conditions

Protein in PBS at pH 7.4 was used for conjugation and introduced the isocyanate in dry acetone solvent to prevent hydrolysis and polymer formation before the conjugation reaction. Under test tube conditions isocyanates would have the following order of reactivity: sulfhydryls >> primary amines > secondary amines > hydroxyls²⁰ as shown in Scheme 3.1.



Scheme 3.1: The highly reactive nature of isocyanates is highlighted in this scheme.

3.2.4 Post Reaction Sampling

The use of quenching agents or dialysis to stop the reaction may also contribute to variability in the structure of the final antigen. Theoretically, stopping after a certain reaction time may influence degree of conjugation or the quenching agent may ‘cap’ any unreacted functional group on the second reactive isocyanate group, possibly yielding a unique and possibly irrelevant antigenic determinant⁴⁰. Similarly, dialysis with ammonium bicarbonate, for example, may result in formation of a product capped with urea⁵². In our conjugations, it was felt there was no need to quench or stop the reaction because the diisocyanates are readily hydrolyzed in aqueous environment⁸. In the reaction mixture hydrolysis was a competing reaction with conjugation, hence the need for a slow controlled addition of the isocyanate was used to increase conjugation as opposed to hydrolysis. Post reaction stirring was done for 15mins to completely evaporate the acetone solvent used to deliver the isocyanate. Dialysis was also done to remove any soluble toluene diamine (TDA) using a molecular cut-off of 14 kDa since our conjugates were greater than 60 kDa and a molecular cut-off of 3.5 kDa for the lysozyme (Mwt 14.6 kDa) conjugate since it is a smaller protein. After dialysis, the conjugates were sterile filtered at 45 µm pore size to remove any precipitated TDA formed from the hydrolysis of dNCOs with the aqueous environment.

3.3 Reaction Product Characterization

3.3.1 Bradford Protein Quantification Assay

The Bradford protein assay was used to quantify the amount of protein after conjugations. Four spectroscopic methods are routinely used to determine the concentration of protein in solution⁸⁴. These include measurement of the proteins by intrinsic UV absorbance or by reaction to protein amino acids producing color/absorbance changes (Lowry assay⁸⁵, the Smith Cooper/Bicinchonic assay and the Bradford dye assay⁶⁵).

The Bradford assay is fairly accurate and fast as compared to the other assays. It is a colorimetric assay based on an absorbance shift in the Coomassie dye from the previously red form of the Coomassie dye to blue following binding to a protein. The bound form of the dye has an absorption spectrum maximum at 595 nm, while the unbound form is red at 465 nm⁶⁵. The Bradford assay is less susceptible to interferences by various chemicals that may be present in the sample⁸⁶. The amino acids of interest are the hydrophilic arginine and hydrophobic phenylalanine, tryptophan and proline (aromatic amino acids)⁸⁶.

The method was not able to quantify non-polar or basic proteins, such as keratin and lysozyme respectively, as these are insoluble in physiological buffers, because the Bradford reagent is water-soluble⁸⁷. The assay is less accurate for basic or acidic

proteins. The assayed protein concentrations were approximately 5 mg/mL (see Table 3.1).

3.2.2 Extent of Conjugation

The extent of adduction of the conjugates was analyzed by the two methods presented in Table.3.1. Using TNBS we found that there was conjugation. However, some of the conjugates gave a negative TNBS assay result. The TNBS reagent assesses primarily chemical adduction with primary amines of amino acids⁵⁹ on the surface of the protein. Additional analyses were also performed using mass spectrometry. For a molar ratio of 1:40 HSA:dNCO, the TNBS assay indicated 10 bound TDI adducts which is in agreement with the 11 amine residues available for binding on the surface of HAS. Mass spectrometry, however, detected 23 adducts/TDI moieties. This difference may be attributed to dNCO reacting with nucleophilic groups other than amines with extensive cross-linking and polymerization of the dNCO causing it to have a high number of conjugations adducts. Binding of one isocyanate to the protein and hydrolysis of the other on a dNCO will also result in no net change in the number of available primary amines and no decrease in TNBS absorbance.

Spectral Scan of HSA -TNBS

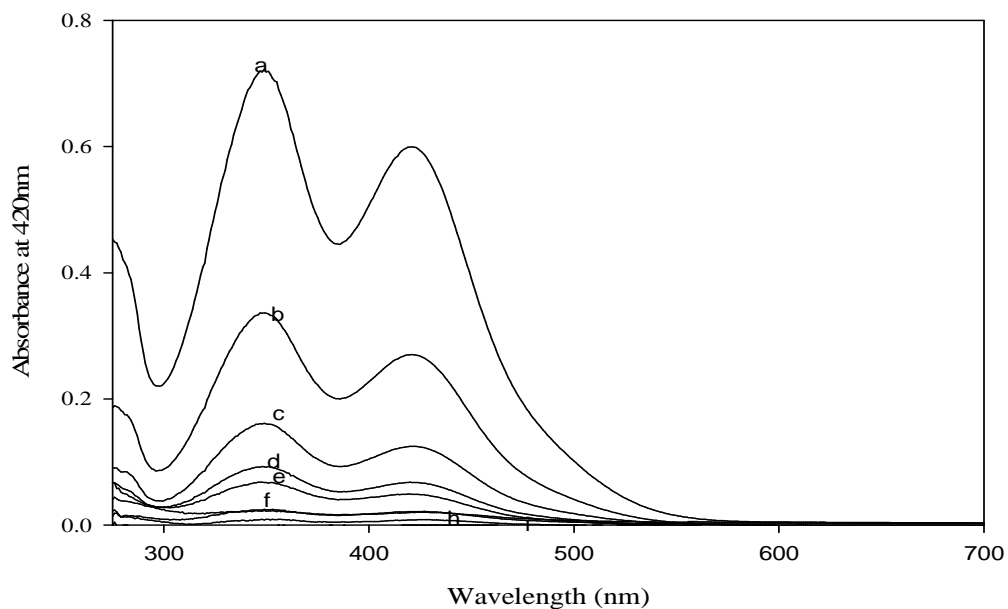


Figure 3.1: Spectral scan of TNBS-HSA complex. [HSA] $\mu\text{g/mL}$ a) 120 b) 60 c) 30 d) 15 e) 7.5 f) 3.75 g) 1.875 h) 0.9375. Scan showing HSA-TNBS complex spectra. A wavelength of 420 nm was used in the TNBS assay to measure amine reactivity and extent of reaction, because there is no solvent or reactant interference.

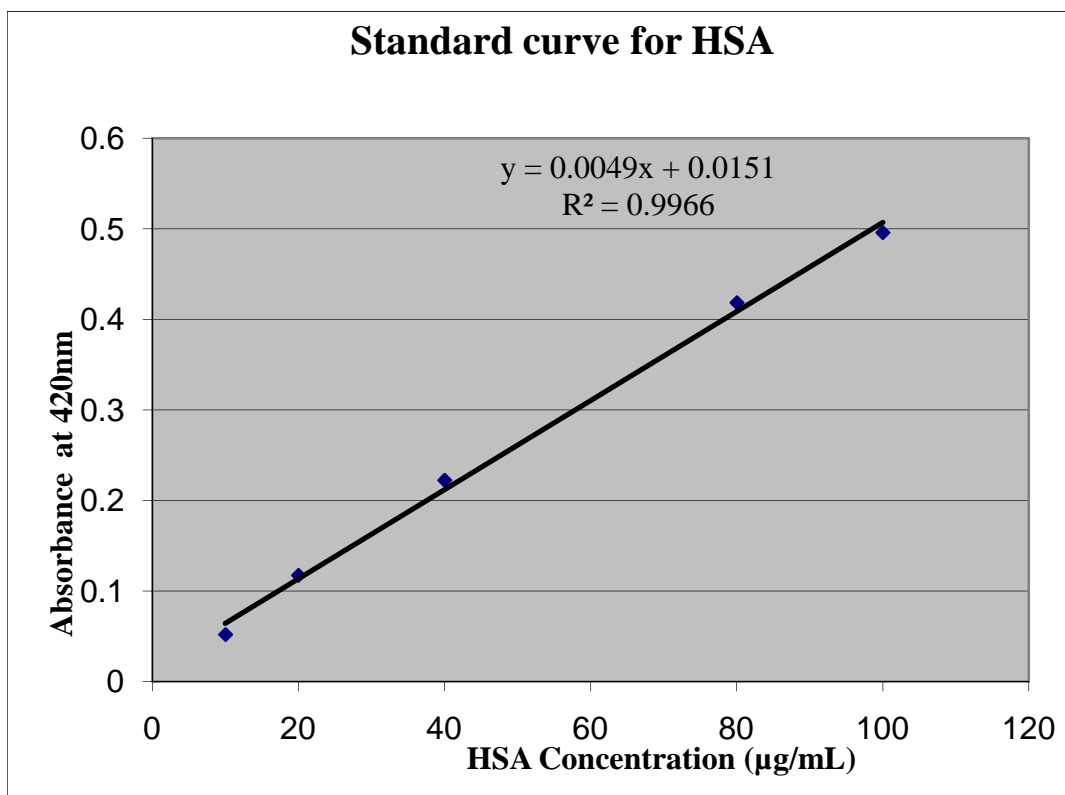


Figure 3.2: HSA standard curve used in the TNBS Assay. Samples whose absorbance at 420 nm is within the linear range of the standard curve were assayed, their absorbance values compared with the curve and used to calculate the extent of conjugation in relation to HSA.

Table 3.1: Characterization of protein adducts using spectroscopic procedures.

Protein	Measured Concentration (mg/mL)	% Substitution (Using equation 3.1)	Number of bound Isocyanate by TNBS	Number of bound Isocyanate by Mass spectrometry
HSA	5.7	0	0	
2,4 TDI-HSA 1:40	4.4	87	10	23
2,4 TDI-HSA 1:10	4.41	90	11	
2,6 TDI-HSA 1:40	4.3	85	2	43
2,6 TDI-HSA 1:10	3.7	69	9	
2,4;2,6 TDI-HSA(pure)	4.1	85	9	-
2,4;2,6-TDI-HSA (industrial)	4.5	82	9	-
2,4 1 st ;2,6 2 nd TDI-HSA	5.6	70	8	3
2,6 1 st ;2,4 2 nd TDI-HSA	5.4	61	5	3
Collagen	0.1	0	-	0
2,6 TDI-Collagen	-	61.8	-	-
2,4 TDI-Collagen	-	-	-	-
Keratin	-	0	-	-
2,4 TDI-Keratin	-	-	-	-
2,6 TDI-Keratin	-	-	-	-
Lysozyme	-	0	-	0
2,4-TDI-Lysozyme	-	-	-	3
2,6 TDI-Lysozyme	-	-	-	-
HDI-HSA	4.5	77	8	26
MDI-HSA	5.0	66	7	25
AHSA	0.2	-	-	-
2,6 TDI-AHSA	-	-	-	-
2,4 TDI-AHSA	-	-	-	-

Protein	Measured Concentration (mg/mL)	% Substitution (Using equation 3.1)	Number of bound Isocyanate by TNBS	Number of bound Isocyanate by Mass spectrometry
KLH	6.1	0	-	0
2,4 TDI-KLH 1:40	5.2	84	-	-
2,4 TDI-KLH 1:10	5.6	77	-	-
2,6 TDI-KLH 1:40	5.2	84	-	-
2,6 TDI-KLH 1:10	5.9	84	-	-
PTI-HSA 1:40	2.5	21	2	3
PTI-PTI 1:10	4.2	12	1	-
OTI-HSA 1:40	3.1	40	4	-
OTI-HSA 1:10	3.8	26	3	-
PI-HSA 1:10	4.0	10	1	-
PI-HSA 1:40	3.0	14	2	5
2,4 TDI-MSA	3.4	53	-	15
2,6 TDI-MSA	3.3	30	-	5
2,4 TITC-HSA	3.4	39	4	4
2,6 TITC-HSA	2,6	37	4	2
2,3 DMPI-HSA	4.6	83	9	25
2,5 DMPI-HSA	3.1	81	9	27
3,5 DMPI-HSA	3.3	73	8	22

The MALDI mass spectra in Figure 3.3 show typical scans collected for the native protein and its conjugates. The determination of bound dNCOs was calculated by mass difference by subtracting the unconjugated carrier protein mass from the conjugated protein mass. The average molecular weight (MW) of HSA was 66679 amu. The

average MW for 2,4 TDI-HSA was 70743 amu indicating an average of 23 dNCO moles/mole HSA, while that for 2,6 TDI-HSA indicated a 43 dNCO moles/mole HSA. Campo⁴⁴ *et. al* compared HDI conjugations from various groups using different conjugation techniques ranging from 0.3 to 33 dNCO: HSA and in our study we had a range from 0 to 43 dNCO:HSA by mass spectrometry.

The spectra also suggested dimer formation as there were peaks at around 120 000 amu (data not shown), an observation corroborated by protein blots shown in Figure 3.4 . This can be due to one dNCO cross linking two molecules of HSA or due to gas-phase dimerization of the proteins . There was no difference in conjugation extent between pure 2,4/2,6 TDI- and the industrial 2,4/2.6 TDI-HSA conjugates.

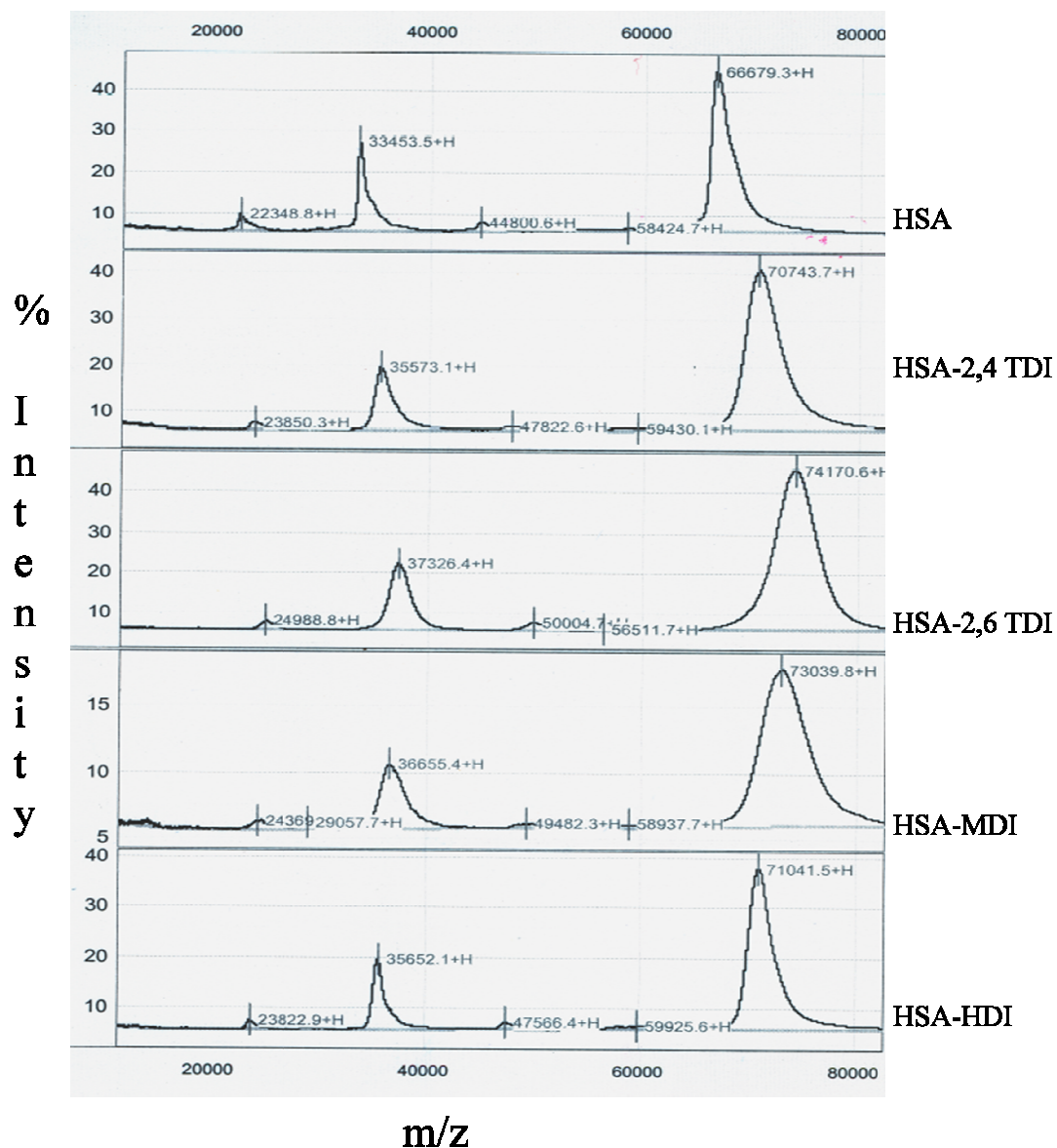


Figure 3.3: Representative mass spectra for protein characterization. Typical mass spectra of HSA control; 2, 4 TDI-HSA; 2,6 TDI-HSA; MDI-HSA and HDI-HSA. See Appendix A for some of the conjugate MALDI spectra.

3.3.3 Conjugate Analysis

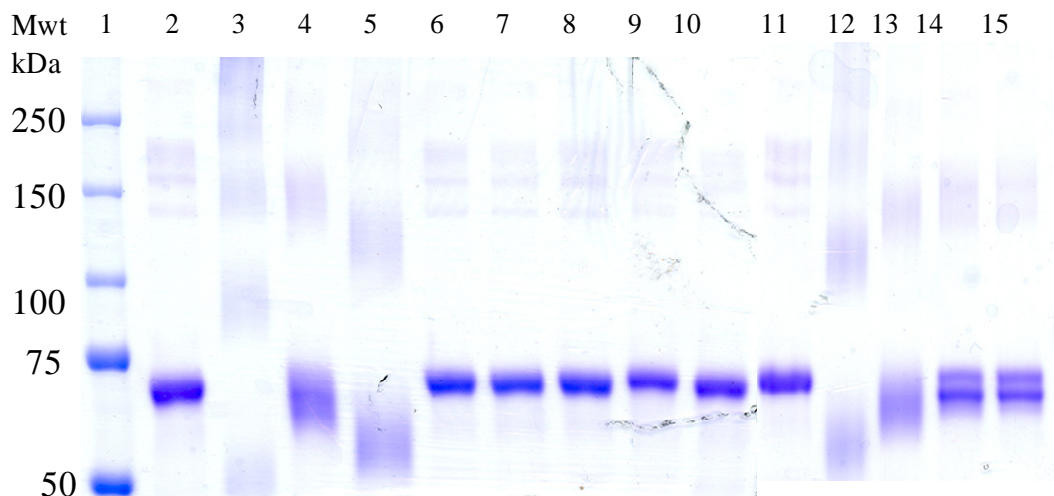


Figure 3.4: 6% SDS-PAGE Protein blot of conjugates. Lane 1, Precision Plus Protein™ standard (Bio-Rad) molecular weight marker; Lane 2, HSA; Lane 3, 2,4 TDI-HSA; Lane 4, 2,6 TDI-HSA; Lane 5, 2,4:2,6 TDI-HSA; Lane 6, 2,3 DMPI; Lane 7, 2,5 DMPI; Lane 8, 3,4 DMPI; Lane 9, OTI-HSA; Lane 10, PTI-HSA; Lane 11, HSA; Lane 12, MDI-HSA; Lane 13, HDI-HSA; Lane 14, 2,4 TITC and Lane 15 2,6 TITC.

Figure 3.4 is a representative SDS-PAGE Coomassie blue stained protein gel of diisocyanate-, monoisocyanate- and diisothiocyanate- conjugated HSA. The protein stain showed extensive cross-linking of the 2, 4 TDI-HSA and also the other diisocyanates. Minor higher MW protein bands were observed in the unconjugated HSA standard (lane 2). There are upward shifts in the dNCO conjugated HSA in Lanes 3, 4, 5, 12 and 13. This indicates formation of polymers through intermolecular

cross-linking and addition of multiple TDIs to HSA. It is also apparent that inter-molecular cross-linking of dNCS haptenated protein (Lanes 14 and 15) was not as extensive as seen with dNCO. The apparent lower molecular weight of some dNCO-HSA conjugates compared to unconjugated HSA may be due to intra-molecular cross-linking that prevents complete denaturation and an increase in electrophoretic mobility of the conjugates. Monoisocyanates are not capable of cross-linking nucleophilic moieties within or between proteins (Lanes 6-10).

3.3.4: Tandem Mass Spectrometry

Attempts to analyze our conjugates by digestion and tandem mass spectrometry were conducted. This proved to be difficult due to the complex nature of the product formed. Table 3.2 below shows representative data of the possible NCO-HSA digest that we had and the observed shifts in m/z . We then decided to start by using simple peptides and a combination of mono-substituted diisocyanate and diisocyanates to develop methods and databases that we could use for our dNCO-HSA conjugation sites. Figure 3.5 show a MS/MS spectrum from TDI-PI, showing conjugated Lys²³⁶.

Table 3.2. Representative data of the theoretical MS/MS conjugate peptide analysis

m/z	PI	M+2H	1 TDI	Amino acids positions	mc	Peptide sequence
4089.87 9	4208.917	2104.96213	4237.03	301-337	1	ECCEKPLLEKSHCIAEVEN D EMPADLPSLAADFVESK
4037.89 3	4156.930	2078.96893	4185.05	509-543	1	RPCFSALEVDETYVPKEFN A ETFTFHADICTLSEK
3626.80 8	3745.845	1873.42648	3773.96	397-426	1	VFDEFKPLVEEPQNLIKQN C ELFEQLGEYK
3563.86 0	3682.897	1841.95248	3711.01	45-75	1	ALVLIAFAQYLQQCPFEDH V KLVNEVTEFAK
3514.67 2	3633.71	1817.35863	3661.82	131-160	1	DDNPNLRLVRPEVDVMC TA FHDNEETFLK
3407.61 0	3526.647	1763.82758	3554.76	384-413	1	CCAAADPHECYAKVFDEF KP LVEEPQNLIK
3365.68 7	3484.724	1742.86588	3512.84	37-65	1	DLGEENFKALVLIAFAQYL Q QCPFEDHVK
3362.52 2	3481.559	1741.28338	3509.67	311-341	1	SHCIAEVENDEMPADLPSL A ADFVESKDVCK
3059.49 9	3178.536	1589.77208	3206.65	470-496	1	MPCAEDYLSVVLNQLCVL HE KTPVSDR
2917.32 2	3036.36	1518.68363	3064.47	311-337	0	SHCIAEVENDEMPADLPSL A ADFVESK
2859.34 7	2978.384	1489.69588	3006.50	500-524	1	CCTESLVNRRPCFSALEVD E TYVPK

Table 3.2 shows possible theoretical fragments that could have reacted with the NCOs. After digestion and mass spectral deconvolution, a computer program would search for any peaks with mass shifts that would correspond to any of our bound isocyanates. Table 3.2 is representative of expected mass to charge ratio of unconjugated HSA, PI conjugated, TDI conjugated single charged molecule and doubly charged molecule and the positions on the primary structure HSA.

**MS/MS Spectrum of HSA peptide 234-242 “AFKAWAVAR”
modified at Lys²³⁶ with phenylisocyanate**

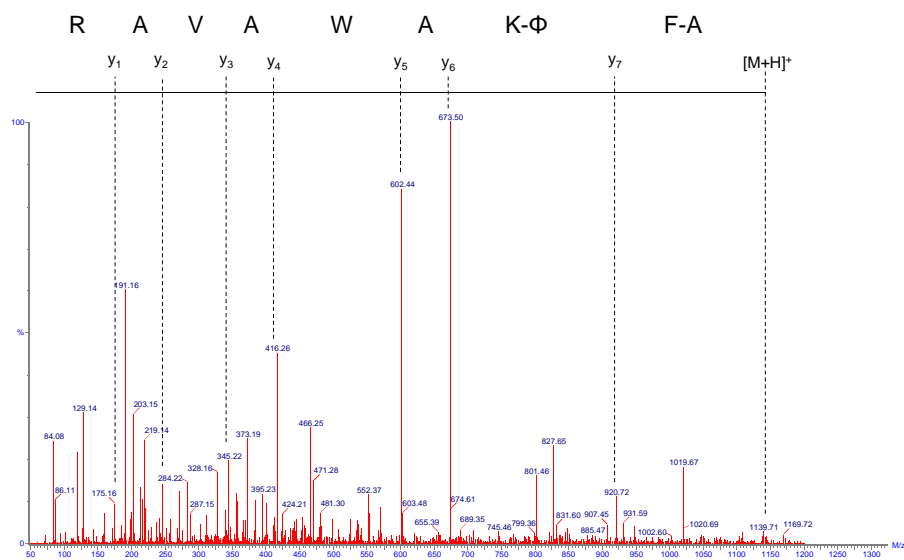


Figure 3.5: Representative spectra showing PI bound to Lys²³⁶.

It has recently been shown that MDI binds to the following lysine residues shown overleaf in Figure 3.6. For PI a different lysine moiety is bound than those found with HDI¹⁵ and MDI⁸⁸.

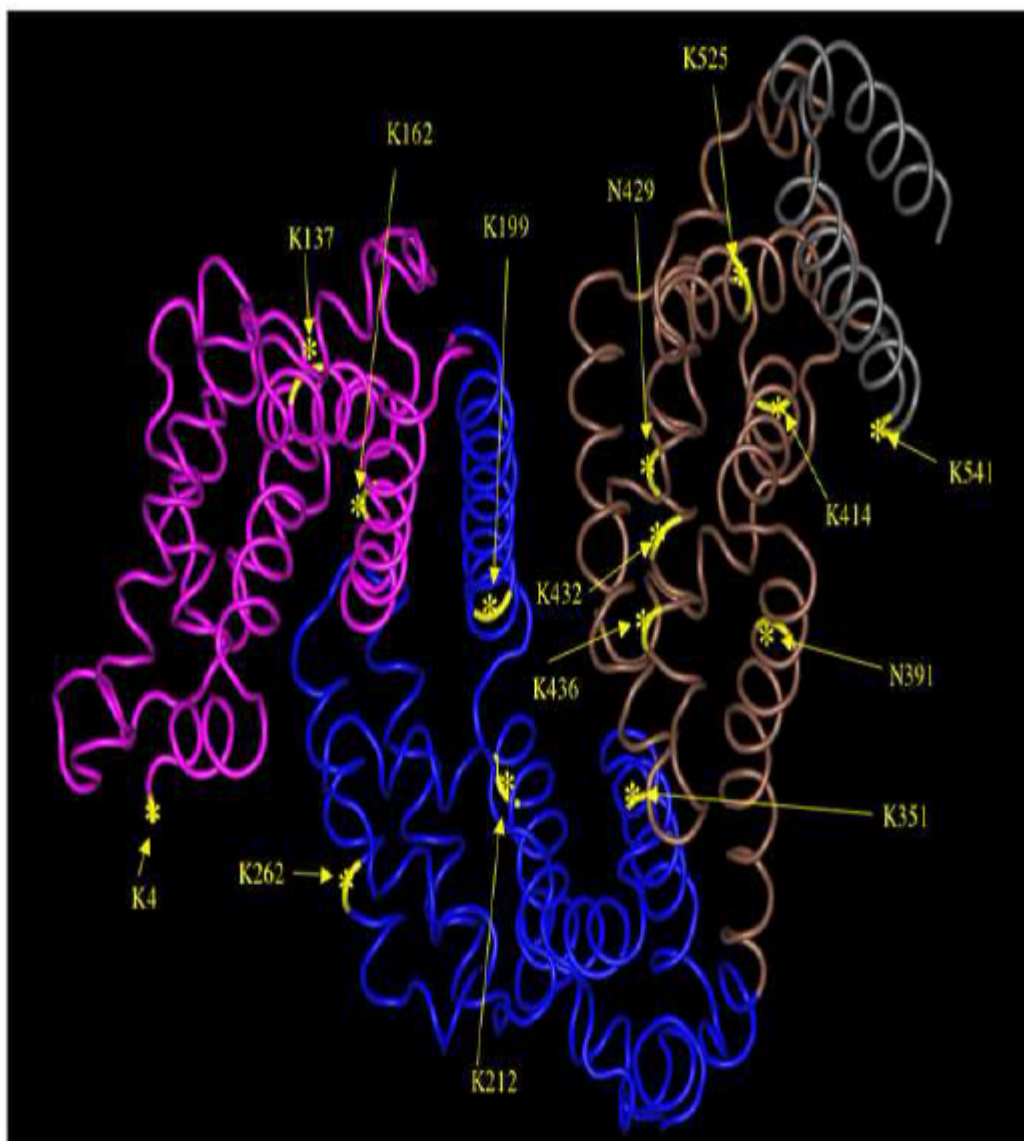


Figure 3.6 : Localization of MDI conjugation sites on human albumin. The sites of MDI conjugation identified by HPLC-MS/MS are highlighted on a 3-D representation of human albumin obtained from the protein/molecular modeling data base, and displayed using the Cn3D program from NCBI⁸⁸.

Conclusion

Identification and characterization of dNCO haptenated proteins from isocyanate exposed workers is important for developing the best diagnostic assays. Diisocyanates may react to multiple nucleophilic moieties within a protein, although the γ -amine on lysines is consistently found as a target. TNBS assay and mass spectrometry techniques employed in this chapter resulted in discordant results as to the number of isocyanates bound. TNBS assay is used to measure loss of primary amine reactivity. For bifunctional electrophiles, such as dNCOs, however, reaction with nucleophilic groups other than amines, extensive cross linking and polymerization of the dNCO, and binding of one isocyanate to the protein and hydrolysis of the other on a dNCO resulting in no net change in the number of available primary amines are all problems with this characterization method. The lack of detecting successful conjugation of keratin may be because it is insoluble in physiological buffers. TNBS, by the way, is water-soluble. Lysozyme is a basic protein and TNBS assay is less accurate for basic or acidic proteins⁸⁷. The extensive cross-linking of proteins is also evident in protein blots where higher molecular weight conjugates were observed. The MALDI-TOF-MS method provides only an average mass shift within a sample, but does not provide information with respect to binding sites, or degree of polymerization. These results also show how difficult it is to analyze and have standard conjugates with defined conjugation reaction ratios⁴⁰. More advanced techniques including QTOF-MS/MS are now being explored^{88,89}.

The complex nature of the products as has been seen in the characterization of the products formed, has been highly problematic. Due to this complexity, we decided to start with binding peptides to NCOs and then monoisocyanates with HSA as a way of developing our methodology and gaining greater insights into the chemistries involved.

CHAPTER 4

STRUCTURAL ELUCIDATION OF ISOCYANATE-PEPTIDE ADDUCTS USING TANDEM MASS SPECTROMETRY

4.1 Introduction

Although several protein targets of diisocyanates, *in vivo*, have been identified, the underlying antigenic forms of the diisocyanate are, as yet, unknown^{16,44,51}. The diverse functional groups present in proteins including amines, amides, thiols, alcohols and carboxylic acids present a large number of potential reaction sites for the isocyanate. However, under physiological conditions, these are limited to N-terminal α -amines, the sulfhydryl group of cysteine, the hydroxyl groups of serine and tyrosine (in hydrophobic pockets only), the ϵ -amine of lysine and the secondary amine of the imidazole ring of histidine³⁵.

Tandem mass spectrometry, in particular collision induced dissociation (CID) of $[M+2H]^{2+}$ ions produced via electrospray ionization (ESI),⁹⁰⁻⁹² is a powerful technique for the determination of not only primary sequence in peptides, but also post-translational modification⁹¹ or chemical adduction. In this study, CID was applied on a high resolution quadrupole time-of-flight (qTOF) mass spectrometer⁹¹ to characterize the adducts produced from the reaction of four commercially available bioactive peptides with four industrially relevant mono- and diisocyanates in an effort

to: a) determine the number, chemical identity, and relative abundance of reaction products observed, and

b) identify the specific binding site of the isocyanate within the peptide. In particular, we have studied adducts formed by 2, 4 and 2, 6 TDI because of the widespread use of these chemicals in manufacturing and their well-documented health effects. Due to the possibility of complex reaction products including inter- and intramolecular cross-linking with the diisocyanates, we have also evaluated the binding chemistry of the monoisocyanate analogues of 2,4 and 2,6 TDI, PTI and OTI. We hypothesize that the binding will be peptide specific and will occur primarily via the n-terminal amine.

4.2 Results and Discussion

4.2.1 MS Analysis of Isocyanate/Peptide Reaction Products

Each of the four peptides (Leu-enk, angiotensin I, FAPP and substance P-amide) were reacted with two monoisocyanates (OTI and PTI) and two diisocyanates (2, 4 TDI and 2, 6 TDI) and the resulting reaction products were analyzed by high resolution qTOF-MS. The relative abundance and accurate mass (± 20 ppm) of the major reaction products was measured in qTOF-MS mode and the identity and relative abundance of the observed reaction products are listed in Tables 4.1-4.3. For clarity, the relative abundance of the unreacted peptide $[M+2H]^{2+}$ ion has been normalized to 1 for each reaction. The only reaction products observed for the monoisocyanates OTI and PTI (Table 4.1) were unreacted peptide $[M+2H]^{2+}$ and adduction of one isocyanate $[M+NCO+2H]^{2+}$ observed at 133.0528 Da higher in m/z. For Leu-enk, angiotensin I

and substance P, OTI was 2-5 times more reactive than PTI. However, in the case of FAPP, PTI was more reactive.

The observed reaction products of the diisocyanate species 2,4 and 2,6 TDI were significantly more varied. A number of reaction products were observed (see Tables 4.2- 4.3) including the anticipated mono-substituted product $[M+dNCO^*+2H]^{2+}$, 148.0634 Da higher in m/z (where the asterisk (*) indicates the product formed by reaction of one isocyanate moiety with the peptide to form a urea and the second isocyanate moiety hydrolyzed to an amine).

Table 4.1. Relative abundances of ortho- and para-toluene isocyanate/peptide reaction products

Peptide	Sequence	M	M+OTI	M+PTI
Leu-enk	YGGFL	1	0.047	0.013
angiotensin I	DRVYIHPFHL	1	8.0	1.4
FAPP	WQPPRARI	1	1.2	3.9
substance P	RPKPQQFFGLM-NH ₂	1	280	120

Table 4. 2. Relative abundances of 2,4-toluene diisocyanate/peptide reaction products

Peptide	Sequence	M	M+TDI*	M+TDI	M+2TDI*	2M+TDI
Leu-enk	YGGFL	1	0.034	--	0.0089	--
angiotensin I	DRVYIHPFHL	1	1.2	0.35	0.56	0.28
FAPP	WQPPRARI	1	0.13	--	0.055	0.094
substance P	RPKPQQFFGLM-NH ₂	1	0.52	3.0	0.29	1.5

** denotes hydrolysis product (amine group at either position 2 or 4)*

Table 4.3: Relative abundances of 2, 6-toluene diisocyanate/peptide reaction products

Peptide	Sequence	M	M+TDI*	M+TDI	M+2TDI*	2M+TDI
Leu-enk	YGGFL	1	0.037	--	0.0011	--
angiotensin I	DRVYIHPFHL	1	2.5	0.13	0.75	0.44
FAPP	WQPPRARI	1	0.14	--	0.040	0.047
substance P	RPKPQQFFGL M- NH ₂	1	4.7	16	1.7	2.8

* denotes hydrolysis product (amine group at either position 2 or 4)

In addition to this product, a second mono-substituted product, $[M+dNCO+2H]^{2+}$, was observed in the case of angiotensin I and substance P, 174.0429 Da higher in m/z.

This product corresponds to both isocyanate functional groups bound to the peptide via ureas, i.e. intramolecular crosslinking. For substance P, this was the dominant reaction product, whereas it was only a minor reaction product (ca. 30% relative abundance) for angiotensin I. Adduction of two hydrolyzed TDI molecules,

$[M+2dNCO^*+2H]^{2+}$, was observed in low relative abundance, as was intermolecular crosslinking of two peptides via TDI, $[2M+dNCO+2H]^{2+}$. Sabbioni and coworkers⁹³ suggested in 2001 that both isocyanate moieties of a diisocyanate would not react at the same time with one or more proteins (e.g. formation of $[M+dNCO+2H]^{2+}$ and $[2M+dNCO+2H]^{2+}$ would not be observed). The current data, however, indicates that these products are formed in aqueous solution, and in the case of substance P, they are the dominant reaction products.

Addition of a second TDI molecule to the peptide is the result of polymerization of isocyanate to the amine of a hydrolyzed diisocyanate, rather than binding of two dNCOs to two different amino acid residues (e.g. side chain adduction). This was

determined based on CID data (described below), as well as the observation of two TDI molecules bound to Leu-enkephalin, which has only one primary amine, the N-terminus.

In all cases, the reactivity of the peptides towards isocyanate showed the trend: substance P > angiotensin I > fibrinectin adhesion promoting peptide > Leu-enkephalin. In general, isocyanates at the 2-position (OTI and 2, 6 TDI) seem to be more reactive than those at the 4-position (PTI and 2, 4 TDI) under the conditions employed for this study. This observation is in good agreement with other reports^{98,101}. Although the isocyanate in the 4- position is roughly four times more reactive than that in the 2- position toward alcohols (during polyurethane production) the 2-position is more reactive toward amines.

4.2.2 Tandem MS Analysis of Isocyanate-Peptide Adducts

Each peptide and its isocyanate adduct product was selected for sequencing by tandem mass spectrometry utilizing Ar collision-induced dissociation in the qTOF mass spectrometer. The MS/MS spectrum of each peptide and its corresponding isocyanate adducts are presented in Figures 4.1-4.6. For each peptide, the tandem mass spectra of the monoisocyanate adducts (OTI and PTI) and the diisocyanate adducts (2, 4 and 2, 6 TDI) were virtually identical in terms of m/z and relative abundance of the observed fragment ions. For simplicity, the fragment ion spectra of the OTI and 2, 6TDI adducts are omitted from Figures 4.1, 4.2, 4.4 and 4.5.

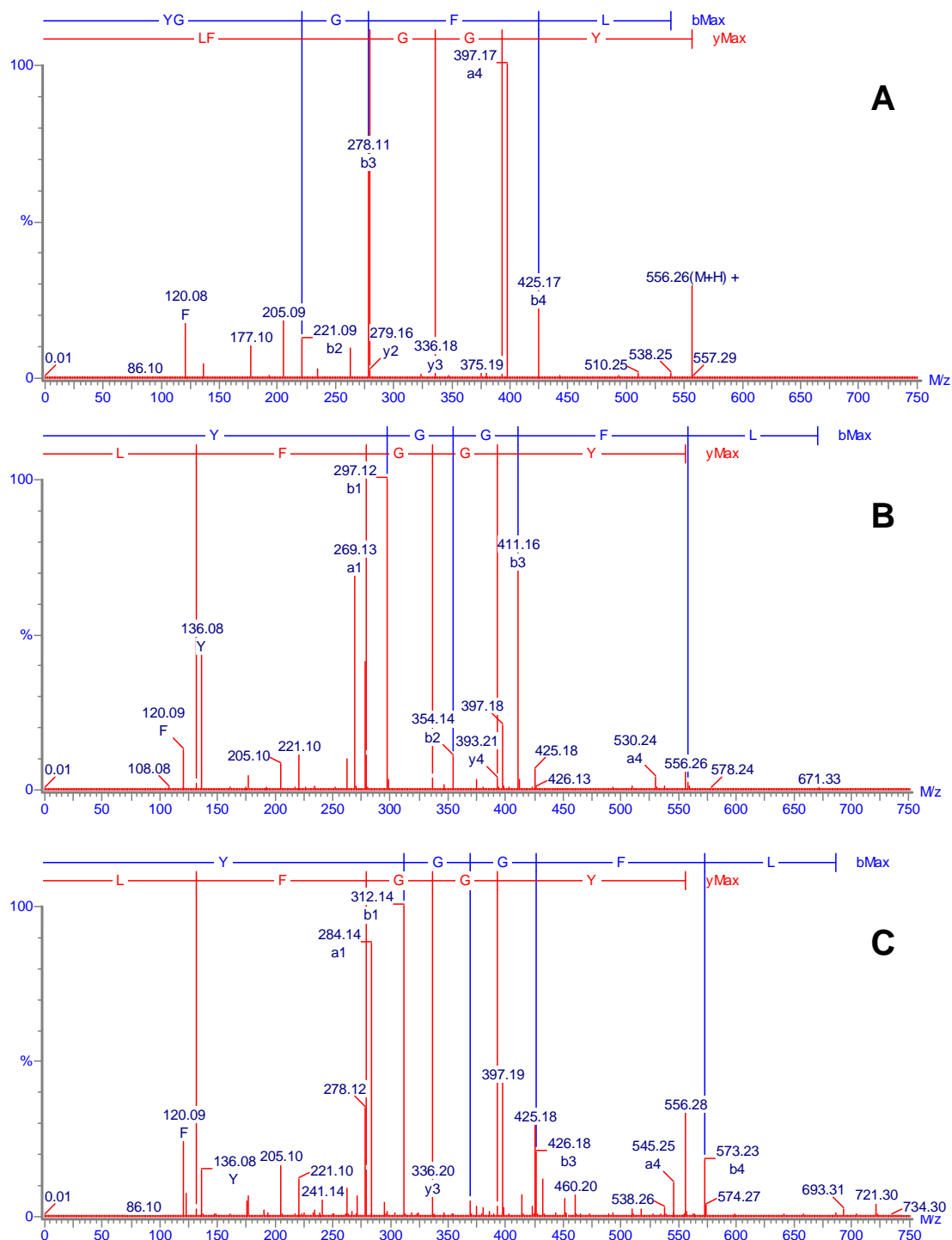


Figure 4.1. Selected tandem mass spectra for Leucine-enkephalin (YGGFL).

A) Unmodified peptide B) PTI adduct C) 2, 4-TDI adduct

Figure 4.1 presents the CID fragment ion mass spectra for Leucine-enkephalin (YGGFL) and adducts produced by reaction with PTI and 2, 4 TDI. The tandem mass spectrum of unmodified Leu-enk (Figure 4.1A) is characterized by abundant a- and b-type fragment ions indicative of N-terminal charge retention. A few low abundance y-type ions, indicative of C-terminal charge retention, are observed. Similarly, the fragment ion mass spectra of the peptide-isocyanate adducts (Figure 4.1B, 4.1C) are characterized by abundant N-terminal charge retention ions. In each case, the base peak in the fragment ion spectrum is of the type $[b_1+NCO]^+$, which provides direct evidence that the isocyanate is bound to the N-terminus of the peptide. Furthermore, the only immonium ion-isocyanate complex observed is the $[Y+NCO]^+$ ion of tyrosine. A complete set of low abundance y_n^+ ions are observed, however, no ions of the type $[y_n+NCO]^+$ are observed, excluding isocyanate adduction from all residues except the N-terminal tyrosine. Loss of the mass of PTI and 2,4 TDI are observed from a-, b- and immonium ions, indicating the urea bond formed when the isocyanate binds to the N-terminus is labile under CID conditions.

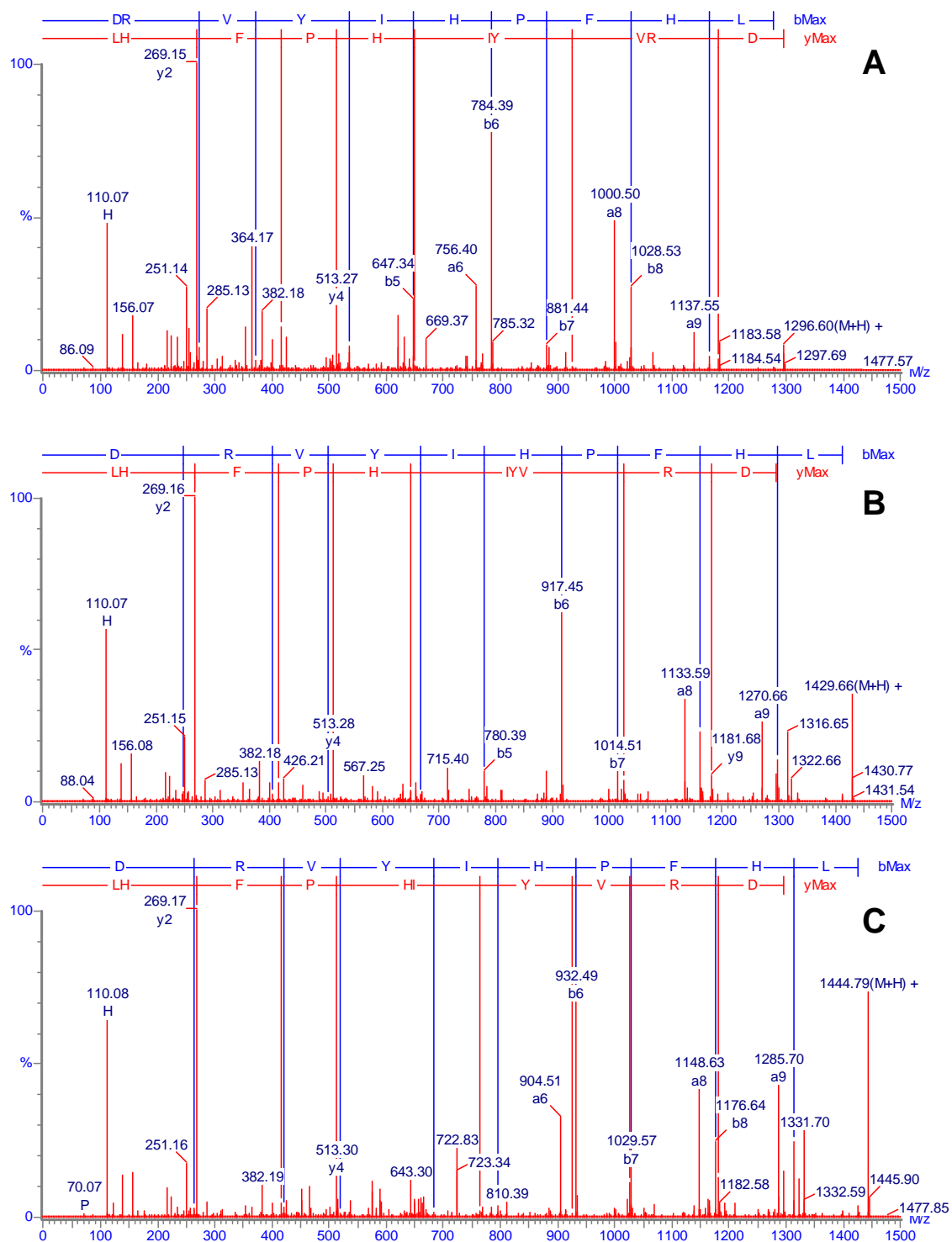


Figure 4.2. Selected tandem mass spectra for angiotensin I (DRVYIHPFHL).

A) Unmodified peptide B) PTI adduct C) 2,4 TDI adduct

The CID tandem mass spectra of angiotensin I (DRVYIHPFHL) and its PTI and 2,4 TDI adducts are presented in Figure 4.2. The tandem mass spectrum of the angiotensin I $[M+2H]^{2+}$ ion (Figure 4.2A) shows abundant fragmentation with both N- and C-terminal charge retention, particularly for ions containing histidine (y_2 and y_4 , for example). The tandem mass spectra of the isocyanate adducts (Figure 4.2B, 4.2C) yield complete sequence information, demonstrating that the isocyanate has bound to the N-terminal aspartic acid residue. Isocyanate is not observed bound to any C-terminal (y -type) fragment ions, nor is it observed bound to the histidine immonium ion. In particular, lack of observation of a prominent $[y_2+NCO]^+$ fragment ion indicates the secondary amine on the side chain of histidine is not reactive with isocyanate under these conditions. We believe the isocyanate is bound to the N-terminal amine rather than the side chain carboxylic acid of aspartic acid because no C-terminal binding of the isocyanate is observed in any of our spectra, and the pK_a of the aspartic acid side chain is similar to C-terminal carboxylic acids, both of which should be deprotonated at non-acidic pH.

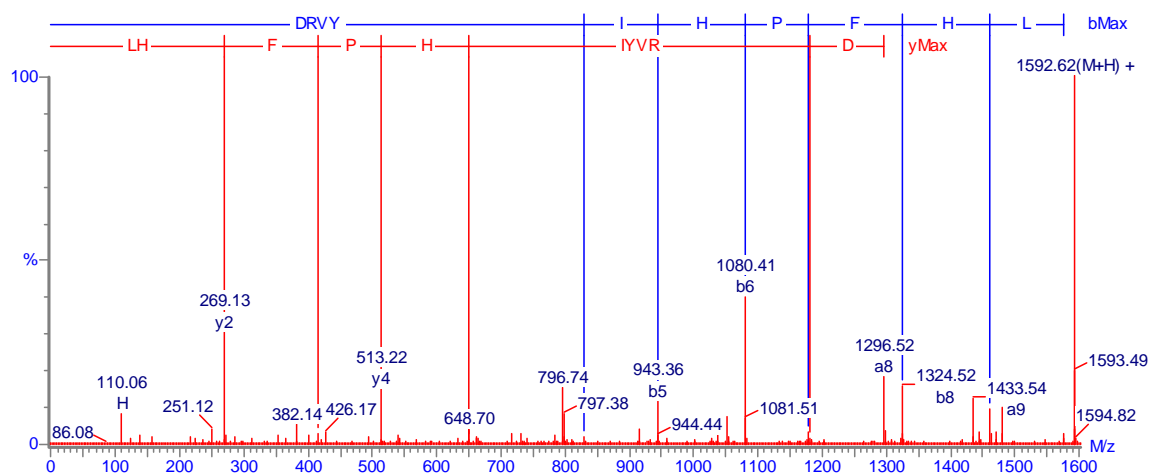


Figure 4.3. Tandem mass spectrum for angiotensin I (DRVYIHPFHL) + 2 2, 4TDI adducts.

For all four peptides examined in this study, we observe an apparent adduction of two TDI molecules (i.e. addition 296.1468 u). In order to determine binding site of second TDI molecule, CID was performed on the $[M+2TDI+2H]^{2+}$ ion of angiotensin I (Figure 4.3). The tandem mass spectrum (in particular the $[y_9+2TDI]^+$ ion) unambiguously identifies the binding site of the second TDI molecule as the N-terminus; in other words, the TDI molecules are polymerizing at the N-terminus via a urea, rather than reacting with a side chain elsewhere within the peptide.

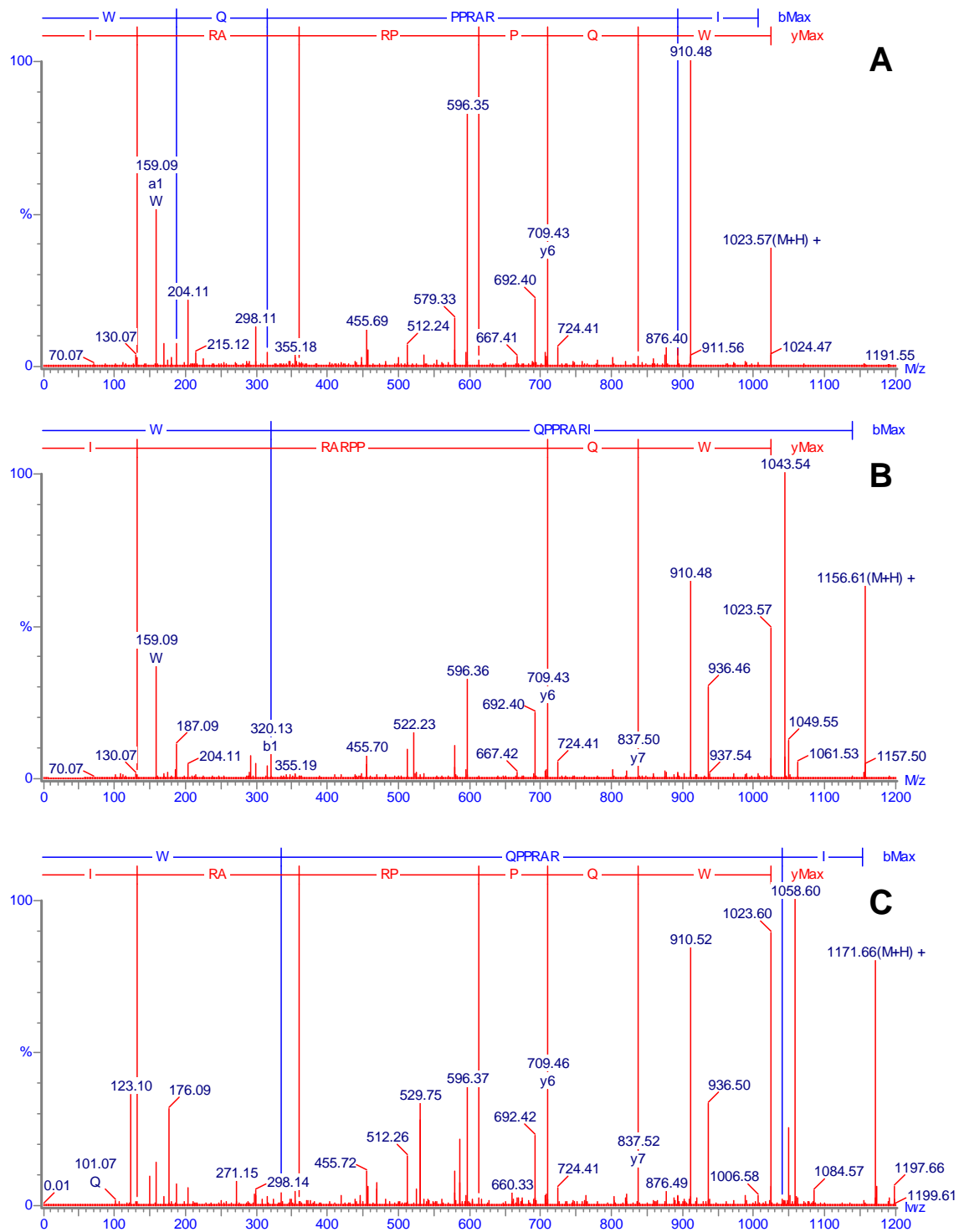


Figure 4.4. Selected tandem mass spectra for fibronectin adhesion promoting peptide (WQPPRARI).A) Unmodified peptide B) PTI adduct C) 2, 4TDI adduct

Figure 4.4 presents the CID tandem mass spectra for fibronectin adhesion promoting peptide (WQPPRARI) and its PTI and 2,4 TDI adducts. The tandem mass spectrum of the FAPP $[M+2H]^{2+}$ ions (Figure 4.4A) shows predominantly y-type ions indicative of C-terminal charge retention. This is not an unexpected result, given the strongly basic residue arginine at positions 5 and 7 within the peptide. Abundant loss of NH_3 from fragment ions is observed, consistent with the tandem mass spectra of other peptides containing Arg, Lys, Gln and Asn. In addition, a strong $[b_7+H_2O]^+$ ion is observed, as has been well documented for peptides with non-C-terminal protonated side chain residues such as Arg, Lys, and His.⁹⁴ From the tandem mass spectrometry data (Figures 4.4 B, 4.4 C) the isocyanate adducts may be unambiguously assigned to the N-terminal tryptophan residue. No ions of the type $[y_n+NCO]^+$ are observed, in particular, the lack of $[y_2+NCO]^+$ and $[y_4+NCO]^+$ indicate that isocyanate does not react with the side chain amine of arginine residues. Further evidence of binding to N-terminus is obtained through the observation of the $[Y+NCO]^+$ immonium ion and an unusual b_1 ion. Formation of acylium b_1 ions is generally considered to result in the loss of CO to form the stable a_1 immonium ion, however, a number of reports have described the formation of stable, cyclic b_1 ions^{95,96}.

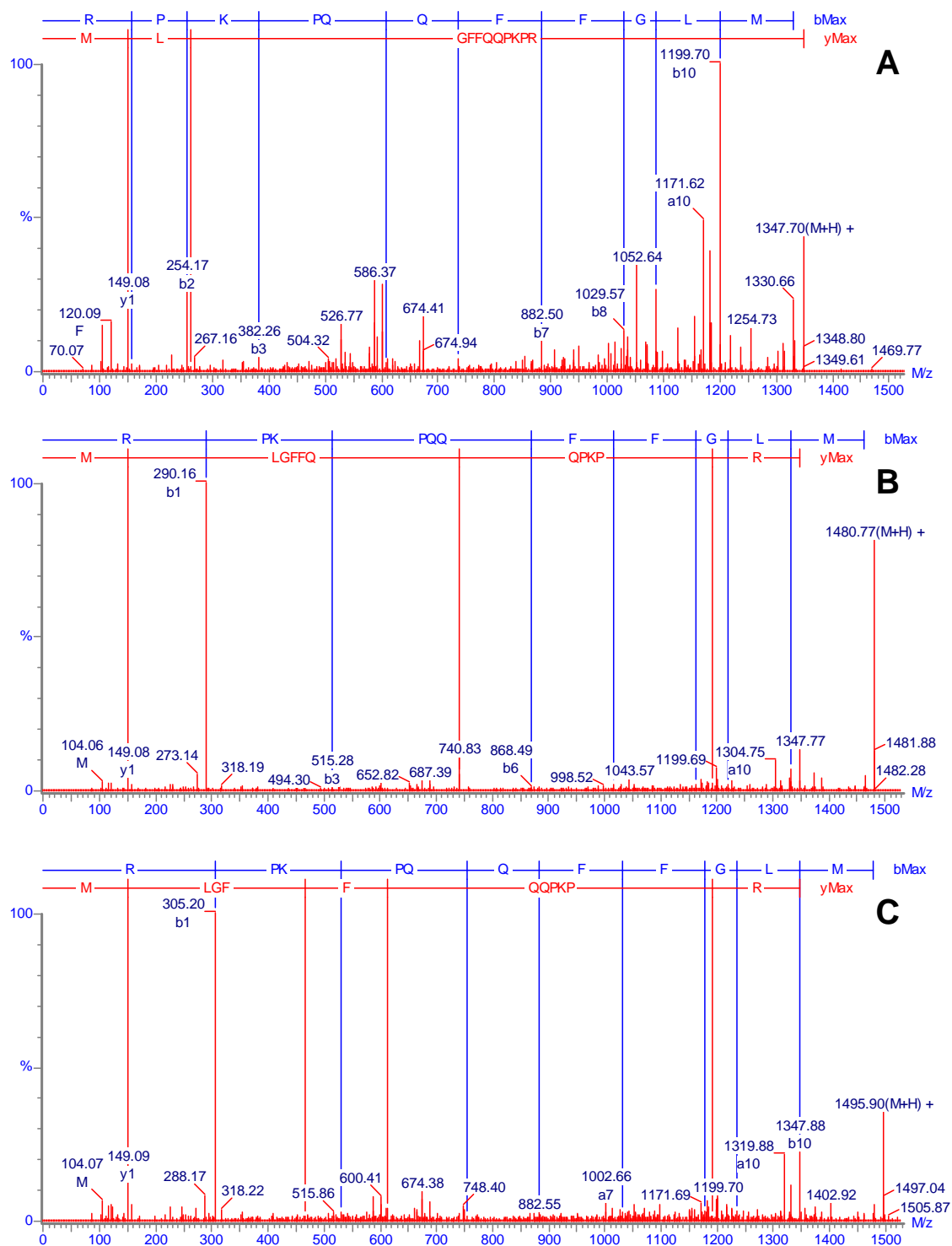


Figure 4.5. Selected tandem mass spectra for substance P-amide (RPKPQQFFGLM-NH₂).

A) Unmodified peptide B) PTI adduct C) 2, 4 TDI adduct

The tandem mass spectrometry data for substance P amide (RPKPQQFFGLM-NH₂) and its PTI and 2,4 TDI adducts is presented in Figure 4.5. Fragmentation of the $[M+2H]^{2+}$ ion (Figure 4.5A) is characterized by abundant a- and b-type ions, internal fragment ions containing lysine, and loss of NH₃. C-terminal y-type ions are observed in low relative abundance. The isocyanate adducts are localized to the N-terminal arginine residue (Figure 4.5B, 4.5C), as evidenced by $[a_1+NCO]^+$ and $[b_1+NCO]^+$ fragment ions. No $[y_n+NCO]^+$ ions are observed. Of particular note, $[y_9+NCO]^+$ (Lys-containing) and $[y_1+NCO]^+$ (C-terminal Met amide-containing) ions are absent, suggesting no isocyanate adduction at either the C-terminal amide or the lysine side chain at position 3. The most abundant product of the reaction of diisocyanates (2,4 TDI and 2,6 TDI) with substance P is the $[M+dNCO+2H]^{2+}$ adduct resulting from intra-molecular cross linking.

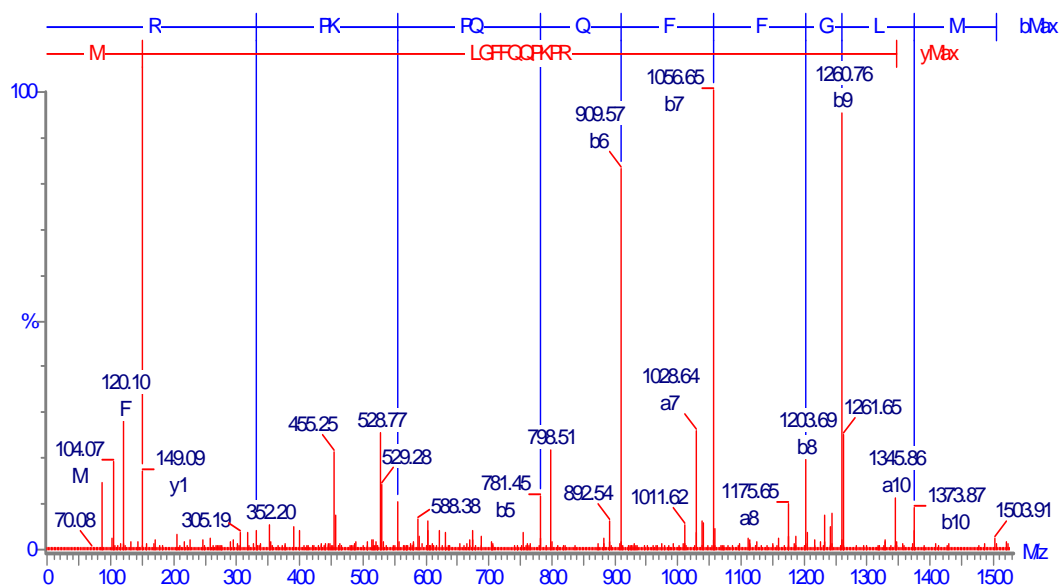


Figure 4.6. Tandem mass spectrum for substance P-amide (RPKPQQFFGLM-NH₂) + 174 u adduct.

The CID fragment ion spectrum of this ion is presented in Figure 4.6. The fragmentation of this ion is similar to that observed for the non-cross linked species in Figure 4.5C. The 174.0429 u adduct from the diisocyanate is located on the N-terminal arginine, presumably bound to the N-terminus and the primary amine on the side chain of arginine. A very low relative abundance of 174 u adduct is observed for the reaction of angiotensin I with diisocyanates (see Tables 4.2, 4.3) which has an arginine residue at position two. Intramolecular cross linking via diisocyanates with arginine residues further removed from the N-terminus (FAPP has arginine at positions 5 and 7) is not observed. We interpret this data in aggregate to indicate diisocyanate rapidly binds to the N-terminal amine and may subsequently react with the side chain amine of a residue in close proximity. We interpret the relative

abundance of these products to indicate that the kinetics of the subsequent side chain reaction are fast with respect to hydrolysis if the arginine is at position one, but slow relative to hydrolysis if the arginine is removed to position two.

Taken as a whole, the present experiments support the findings of Stark⁹⁷ who found that the rate of reaction of isocyanates with amino groups is related linearly to pKa. That work suggested that at pH 7 and below, the N-terminus of peptides and proteins would react approximately 100 times faster than the ϵ -NH₂ group of lysine. Furthermore, Stark suggested the reaction proceeds via the uncharged -NH₂ species, rather than the -NH₃⁺ ion. Similarly, Mason and Liebler⁹⁸ used phenyl isocyanate at pH 8.0 to label the N-termini of peptides generated by proteolytic digests. Other reports^{34,35} have suggested that the N-terminus of proteins (particularly albumin and hemoglobin) is a potential site of diisocyanate adduction, and the results presented here demonstrate conclusively on the basis of tandem mass spectrometry data that the N-terminal α -amine of these four model peptides is the site of adduction.

4.3 Conclusions

Analysis of peptide-isocyanate adducts by tandem mass spectrometry reveals that isocyanates bind preferentially to the N-terminus of the four peptides examined under the conditions employed herein. When a peptide with an N-terminal residue containing a side chain amine is reacted with a diisocyanate, intramolecular cross linking with the second isocyanate becomes competitive with hydrolysis. The

reactivity, however, decreases as the residue is displaced further from the N-terminus.

When the isocyanate-peptide reaction is carried out under 1:1 stoichiometric conditions, the extent of reaction depends on both the choice of isocyanate and peptide. In general, the isocyanate in the ortho position seems more reactive than that in the para position. The results of this chapter, when taken in aggregate with those of previous studies, indicate that the N-terminus of proteins is a likely target for adduction in isocyanate-exposed individuals.

CHAPTER 5

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST TOLUENE DIISOCYANATE HAPTENATED PROTEINS

5.1 Introduction

Köhler and Milstein⁹⁹ developed a technique to grow clonal populations of cells secreting antibodies with a defined specificity. Their work introduced hybridoma technology, which entailed fusion of an antibody-secreting cell isolated from an immunized animal with a myeloma cell derived from a of B-cell tumor to have a hybridized cell.

To develop mAbs, animals (mice) are injected with an antigen followed by routine booster injections⁶⁷. These injections usually incorporate an adjuvant. After 2 to 3 injections, a small sample of blood is collected from the immunized animal to determine blood antigen specific antibody titers. Blood antibody titers provide a measure of the animal's immune response to the injected antigen and the need for continued immunization boosters. Upon reaching a predetermined titer of antibodies in the serum, a final injection series is administered without adjuvant, prior to spleen removal and harvest of spleen cells. After removal of spleen, splenocytes are combined with SP2/0 mouse myeloma cells⁶⁷. Cell fusion can be accomplished using electro-fusion or chemical fusion with polyethylene glycol. Following cell fusion, cells are diluted and plated in multiwell tissue culture plates. Screening of hybridoma containing wells

commences 1 to 2 weeks later, or after sufficient growth to near confluence. Cells grown in positive wells are expanded, frozen, and then undergo clonal dilution. For clonal dilution cells from the original fusion plates are transferred to a second multiwell plate, serially diluted, and allowed to grow. Wells with single clonal colonies are re-screened and positive clones are expanded and frozen. Individual clones are used for mAb production using *in vitro*, static cell culture flasks, or (*in vivo*, mouse ascites fluid technique). Production of mAbs ensures a continuous supply of antibodies of defined characteristics that can be selected and extensively characterized from hundreds of clones in a given fusion. It is the absolute specificity of mAbs for a defined epitope that offers a major advantage over polyclonal antibodies where a variety of antibodies are encountered in any given antiserum which will have as broad range of affinities and specificities for different epitopes in a protein sequence.

5.2 Results and Discussion

It is difficult to produce mAbs from species other than mice. Young mice, preferably female since they are less aggressive and give better immune responses than males, are used¹⁰⁰. Young animals are used, because the numbers of fibroblast in the spleen increases with age. Excessive fibroblast in the primary fusion plate can lead to hybridoma cells being out grown by this cellular competitor.

5.2.1 Mice Immunization

Mice were immunized with TDI-conjugated KLH 1:40 ratio, that was prepared for injection by emulsifying the antigen with TiterMax® adjuvant according to manufactures' instruction. TiterMax® was used instead of the traditional Freund's complete adjuvant in order to reduce the potential of pain and distress to the animals. KLH is an immunogenic carrier protein that, *in vivo*, increases antigenic immune responses to haptens and other weak antigens such as idiotype proteins¹⁰¹. KLH is the most widely employed carrier protein for this purpose^{15,55,101}. KLH is an effective carrier protein for several reasons. Its large size and distinct/foreign epitopes generate a substantial immune response, and the abundance of lysine residues for coupling haptens allows a high hapten:carrier protein ratio which increases the likelihood of generating hapten-specific antibodies¹⁰¹. It also shares no antigenic epitopes with mammalian proteins.

An adjuvant is a substance that augments immune responses in a non-specific manner. The most commonly used adjuvants are Friend's complete or incomplete adjuvant. It is water-in-oil emulsion in which killed and dried *Mycobacterium bovis* bacteria are suspended in oil phase¹⁰².

Other adjuvants include aluminum compounds such as aluminum hydroxide gel and other alums such as potassium alum ($K_2SO_4 \cdot Al_2SO_4$) that strongly adsorb protein antigens from solution to form a precipitate. Alum-precipitated proteins are often administered together with killed *Bordetella pertussis* organisms (whooping cough

vaccine)¹⁰³. In recent years, there have been numerous new adjuvants, including pluronic block polymers that give large surface area, such as Titremax B. An adjuvant should be able to provide long-lasting local deposition of the antigen from which the antigen would be released slowly over a long period providing a prolonged, *in vivo*, boosting of the immune system.

The route of immunization depends on the nature of antigen. Intra-peritoneal (IP), intramuscular (IM), intradermal (ID) or subcutaneous (SC) injection can be used. The spleen is the most accessible source of B-lymphocytes for fusion. The number of times one immunizes the mouse prior to fusion will determine the class of antibody that you produce. The length of time between the immunizations also influences the affinity of antibodies produced. This is called affinity maturation. The physiological purpose of antibody production in an animal is to clear the antigen from the body. After some time only high affinity, B cells are clonally expanded. The longer the interval between immunizations the greater the preferential selection of high affinity B cells. In mice, you have to balance the number of immunizations and length of intervals between them to reduce risk of fibroblast accumulation in older animals.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is a common technique used for the detection of specific antibodies, soluble antigens, or surface antigens¹⁰⁴. Factors that have contributed to IgG mAb ELISA

utility include their sensitivity, long shelf-life of reagents, lack of radiation hazards, ease of preparation of reagents, the speed and reproducibility of the assays and the variety of ELISA formats that can be generated. No sophisticated equipment is necessary and ELISA's have a wide range of applications^{105,106}.

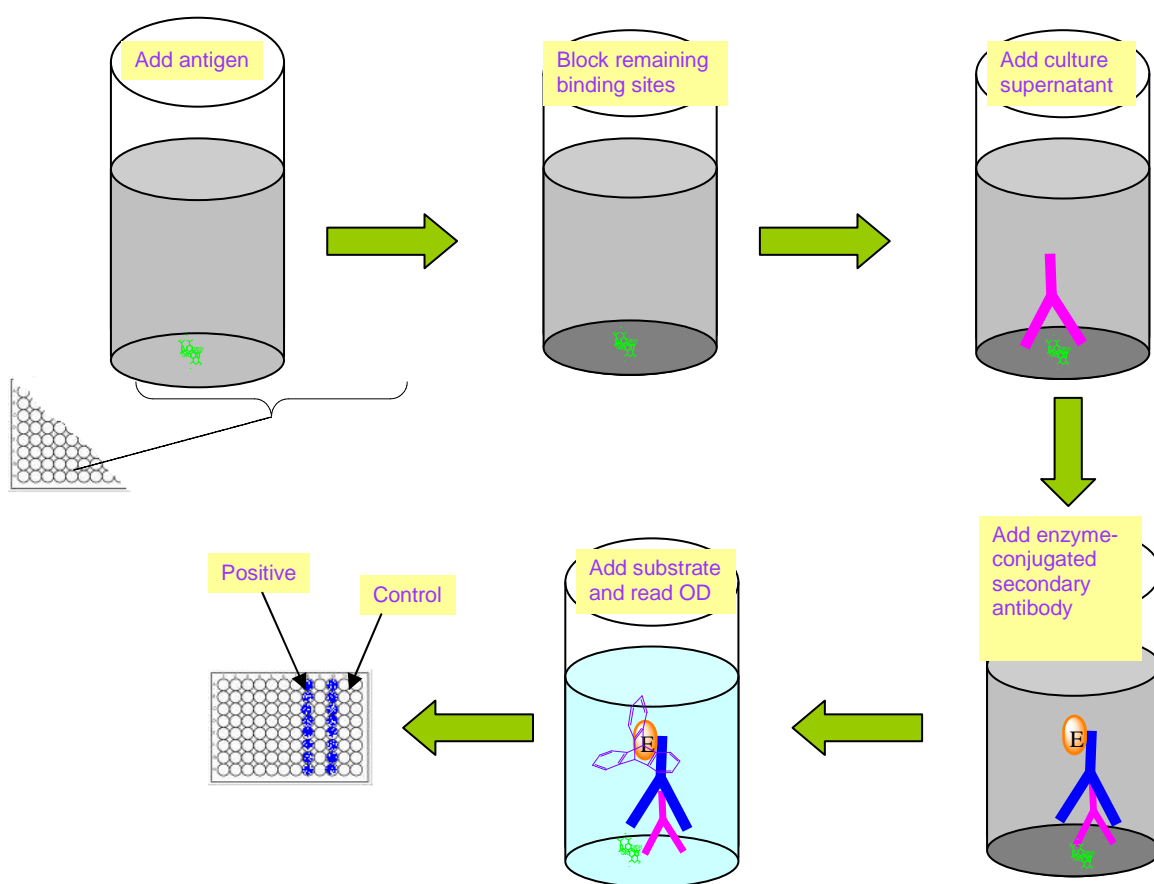


Figure 5.1: ELISA principle for hybridoma screening.

In brief, (Figure 5.1) for ELISA's for screening and reactivity studies, an antigen is

coated onto a plate well. A non specific/target protein such as albumin or casein, is used to block all unbound surfaces (block non-specific antibody binding); culture supernatant fluid with potential antibody of interest is added and incubated until equilibrium is reached (2 hrs at RT). Unbound antibody is removed by washing. An enzyme labeled detection anti-mouse-IgG is added, incubated and unbound labeled IgG removed by washing. The enzyme substrate is added and plate incubated. Color development is monitored spectrophotometrically and is directly proportional to both the amount of labeled antibody bound and to the hybridoma antigen-specific antibody

5.2.2 Screening of Mice for Antibody Production

After several weeks of biweekly immunizations, blood samples were obtained from mice for measurement of specific IgG levels. TDI-specific antibody titer was determined by ELISA, using 2,4/2,6TDI-HSA, carrier protein (HSA) and also the protein with which it was immunized against i.e. 2,4/2,6TDI-KLH. A 1:40 ratio was used as the screening antigen ratio because, according to Table 5.1 below, using pooled human sera from individuals with known IgG titers against TDI, MDI and HDI, the reactivity of human sera increases with increase in conjugation ratio. Wisnewski and colleagues⁸⁸ also found out that the 1:40 conjugation ratio with MDI reacted the most with human sera from a different population exposed to MDI.

Table 5.1: Pooled human sera reactivity with various protein conjugation ratios

Protein	Conjugation ratio	Optical density at 405nm
HSA		0.10
2,4 TDI-HSA	1:40	1.608
	1:10	1.120
2,6 TDI-HSA	1:40	1.704
	1:10	0.408
2,4;2.6 TDI-HSA (mix)	1:40	1.562
	1:10	1.316
PI-HSA	1:40	0.123
	1:10	0.083
OTI-HSA	1:40	1.831
	1:10	0.425
PTI-HSA	1:40	0.515
	1:10	0.108

*The pooled human sera were a donation from Dr. Zana Lummus (University of Cincinnati).

If the antibody reactivity of the 2,4/2,6 TDI-HSA had a titer optical density (OD) value of approximately 1 for the 1:100000 serum dilution, cell fusion was performed.

If the titer was low, $OD < 1$, the mouse was given booster immunizations until an

adequate response was achieved, as determined by repeated blood sampling. When the antibody titer was high enough, the mice were boosted by injecting antigen, IP without adjuvant, 3 days before fusion (approximately, 2 weeks after the previous immunization). The final boost was given 3 days before fusion to ensure the presence of many B immunoblast (antigen activated B cells that are in division), which are the most successful fusion partners for myeloma cells. The mice were euthanized and their spleens removed for *in vitro* hybridoma cell production.

For TDI- vapor exposure immunization, five mice were exposed to TDI vapor for 4 hr/day, for 12 consecutive work days. Lymph nodes and spleens were collected 24 hrs following the final exposure. Spleens and lymph nodes were removed aseptically and lymphocytes harvested after lysing red blood cells by osmotic shock. Hybridomas were produced following standard techniques as previously described ⁶⁷

Table 5.2: Immunization Titers. Mean replicate OD_{405nm} value.

Mouse #	2,4-TDI-HSA					2,6-TDI-HSA				
	Prebleed	Bleed 1	Bleed 2	Bleed 3	Terminal bleed	Prebleed	Bleed 1	Bleed 2	Bleed 3	Terminal bleed
1R	0002	0.009	0.057	0.770	1.023	0.003	0.003	0.006	0.175	1.363
2R	0.008	0.003	0.474	1.285	1.325	0.009	0.012	0.005	1.354	1.891
1L	0.002	0.006	0.036	1.025	2.53	0.048	0.014	0.005	0.384	1.236
2L	0.030	0.002	0.135	0.697	1.692	0.024	0.006	0.041	0.127	1.154
0	0.005	0.010	0.106	1.99	2.217	0.004	0.009	0.009	0.700	1.857

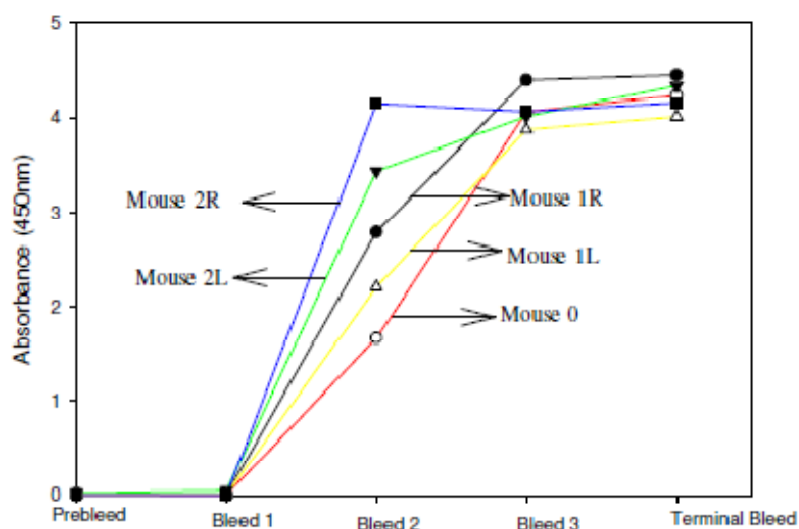


Figure 5.2: Graph showing titer increase for 2, 6 TDI-KLH immunized mice.

Table 5.2 and Figure 5.2 show the increase in the anti TDI-HSA titers with time. Our immunization titers showed a significant increase in 2,4-TDI-HSA specific antibodies for all the mice. Reactivity towards 2,6-TDI-HSA was lower compared with the 2,4-TDI-HSA titers (Table 5.2).

The bleeds were characterized in terms of their reactivities as shown in Table 5.3 below.

Table 5.3: Polyclonal cross reactivity after 3rd immunization

	Mouse #	KLH	2,4/2,6TDI-KLH	HSA	2,4TDI-HSA	2,6TDI-HSA	MSA	2,4TDI-MSA	2,6TDI-MSA
2,4 TDI-KLH	1R	3.920	4.091	0.016	1.023	0.019	0.185	0.672	0.198
	2R	4.105	4.231	0.012	1.325	0.055	0.175	3.150	0.183
	1L	3.863	3.930	0.014	2.53	0.048	0.171	0.463	0.165
	2L	3.914	4.101	0.013	1.692	0.033	0.167	1.342	0.158
	0	4.231	4.254	0.026	2.217	0.586	0.166	0.936	0.136
2,6 TDI-KLH	1R	3.856	3.777	0.009	0.123	1.363	0.213	0.254	0.956
	2R	3.456	4.064	0.024	0.215	1.891	0.232	0.213	1.325
	1L	3.541	3.950	0.015	0.145	1.236	0.184	0.256	1.569
	2L	4.213	4.024	0.020	0.245	1.154	0.240	0.236	1.325
	0	4.01	3.766	0.036	0.254	1.857	0.156	0.198	1.895

The polyclonal sera had high titers to KLH alone since we immunized the mouse using conjugated KLH. There were very high specific titer responses to the immunizing isomer. The MSA titers were higher than HSA titers due to auto antibodies since the sera was derived from mice. Table 5.3 shows the cross reactivity screening of the polyclonal antibodies. The results show that our polyclonal antibodies are carrier protein independent and specific to the isocyanate bound protein only as evidenced by 2,4/2,6 TDI-HSA and 2,4/2,6 TDI-MSA on top of our immunizing antigen, 2,4/2,6TDI-KLH having the higher. titers. There was insignificant non-specific binding to human or mouse albumin by the polyclonal IgGs.

5.2.3 Hybridoma Production and Screening

Fusing antibody-producing spleen cells, which have a limited life span, with cells derived from an immortal tumor of lymphocytes (myeloma) results in a hybridoma

that is capable of unlimited growth. Hybridoma production is divided into harvest of splenic lymphocytes, myeloma cell production, fusion, cloning and screening.

Myeloma cell preparation.

Myeloma cells are immortal cells that are cultured with 8-azaguanine to ensure their sensitivity to the hypoxanthine-aminopterin-thymidine (HAT) selection medium, which is used subsequently for cell fusion¹⁰⁷. Cell viability of exponentially growing cells was checked microscopically prior to use.

Fusion.

Specific variables considered during the fusion were the fusagen, cell ratio, medium, conditions for achieving contact, time, temperature and processing after the fusion.

The original fusagen used was Sendai virus⁹⁹ but this has now been superseded by PEG, which is safer to use and does not require virus culture. The cheapest, simplest and most reliable method is PEG fusion¹⁰³. Freshly harvested spleen cells and myeloma cells were fused by incubating at a 1:10 ratio of spleen: myeloma cells in the presence of the fusagen in PEG solution for 10 minutes at 37°C, cells were co-pelleted by centrifugation at 3XG following incubation. The myeloma cells are hypoxanthine-guanine phosphoribosyltransferase deficient (HGPRT⁻), and therefore are unable to use the purine salvage pathway when *de novo* purine synthesis is blocked by aminopterin. The unfused spleen cells eventually die because they are not immortal¹⁰⁸. Only the immortalized hybridoma cells survive when incubated in HAT medium.

Table 5.4: Fusion results summary for 2,4 TDI-KLH immunized mice

Mouse	% of wells with growth	Number of clones per well	% of positive clones
1R	0	0	0
2R	87.3	2.2	0.118
1L	97.4	2,41	0.3823
2L	72,4	1.75	0
0	92	2.71	1.25

There was an average growth rate of 87% and 2.3 clones per well and of these, 0.44% of the seeded wells were positive for anti-TDI-protein. The 2,6 TDI-KLH and TDI vapor exposed mice fusions had lower fusion rates . After fusion, some of the hybridomas were not viable and were lost during the screening process. During the fusion process a ratio of 1 myeloma cell for every 10 spleen cells was used and by this alone 90% of the spleen cells are lost. This is the optimal ratio of myeloma/spleenic cells for at higher myeloma concentrations they tend to self-react producing fewer hybridomas. Target clones may be lost due to being out-competed by non-target clones since antibody producing colonies tend to grow slower than those that are not producing antibody. Also not all spleenocytes are our targeted cells. So even if there were a cell colony in the spleen that produced our target antibody specific for 2,4 TDI conjugated proteins, we potentially may have failed to capture it. Hence, the rather remarkably low percentage of successful clones. A total of 59 hybridomas specific for 2,4/2,6 TDI-HSA were produced .

Cloning.

Single clones from positive wells were isolated by by employing the limiting dilution technique, twice. When the cells were first plated out they contained different cells in

a single well, hence after growth there may have been many clones in the well. The specific antibody secreting clone, therefore, was likely to be mixed with clones that were either non-secreting or were producing non-specific antibodies.

Limiting dilution is a method based on Poisson distribution¹⁰⁹. Dilution of cells to an appropriate number per culture plate well can maximize the proportion of wells that contain one single clone. Critical parameters included, counting of cells to obtain approximately 0.8 cells per 100 μ L of medium in a 300 μ L well. Immortalized cells replicated within each well and the number of clones per well counted using an inverted microscope. After 7 days, culture fluid was recovered from each well and screened for specific-TDI-conjugated protein IgG/M. All negative wells were discarded. Cells from positive wells were immediately re-cloned to prevent overgrowth by co-cultured non-secreting hybrids. 20-50% of wells seeded at ~0.8 cells/well exhibited growth. Loss of antibody producing clones was expected between clonings¹¹⁰. Positive wells were re-cloned until each well contains only cells from the same original hybridoma.

Freezing and Recovery of Hybridoma Cell Lines.

Hybridoma cells were suspended in 10% dimethyl sulfoxide (DMSO) in fetal calf serum and frozen rapidly in a dry ice-ethanol and glycerol bath followed by transfer to liquid nitrogen storage¹¹¹. Hybridomas are stable as stored for years and can subsequently be resurrected and cultured for antibody production.

Each task described above can be summarized as in Figure 5.3.

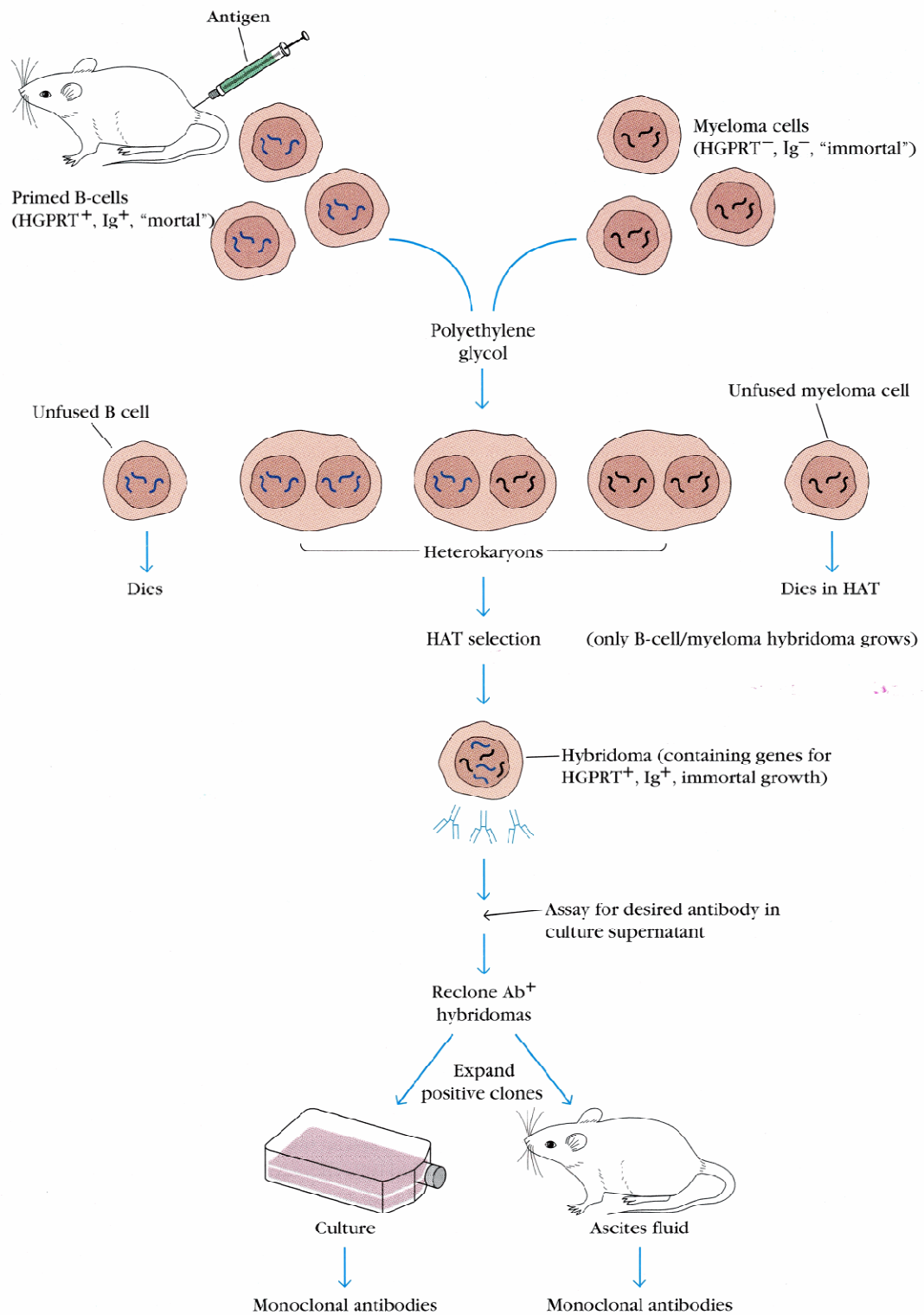


Figure 5.3 : Summary diagram of monoclonal antibody production¹¹².

5.2.4 Antibody Isotyping and Concentration Determination

The monoclonal antibodies were quantified using isotype specific ELISA kits according to the manufacturers' instructions. Antibody concentrations in the culture supernatants fluids ranged from 0.160 $\mu\text{g/mL}$ to 169 $\mu\text{g/mL}$ with an average concentration of 52 $\mu\text{g/mL}$. Seven mAbs reacted with 2,4 TDI –HSA, 1 mAb reacted with 2,6 TDI-HSA only, while 46 were found to react with 2,4 and 2,6 TDI HSA conjugates. Twenty-nine hybridomas were found to be IgG1, 14 IgG2a, 4 IgG2b, 2 IgG3, and 10 IgM. See Table 5.4 for complete list of mAbs produced.

Antibodies are heteromeric molecules consisting of heavy and light chains, each of which contains a variable and a constant region. Heavy chain constant regions include μ , α , $\gamma 1$, $\gamma 2a$, $\gamma 2b$, $\gamma 3$, $\gamma 4$, σ or ϵ depending on species; light-chain constant regions include κ and λ ¹¹². Immunoglobulin heavy constant regions are referred to as isotypes, determine many biological and immunochemical properties of the antibody including complement fixing, binding to Fc receptors and binding to proteins A and G¹¹³.

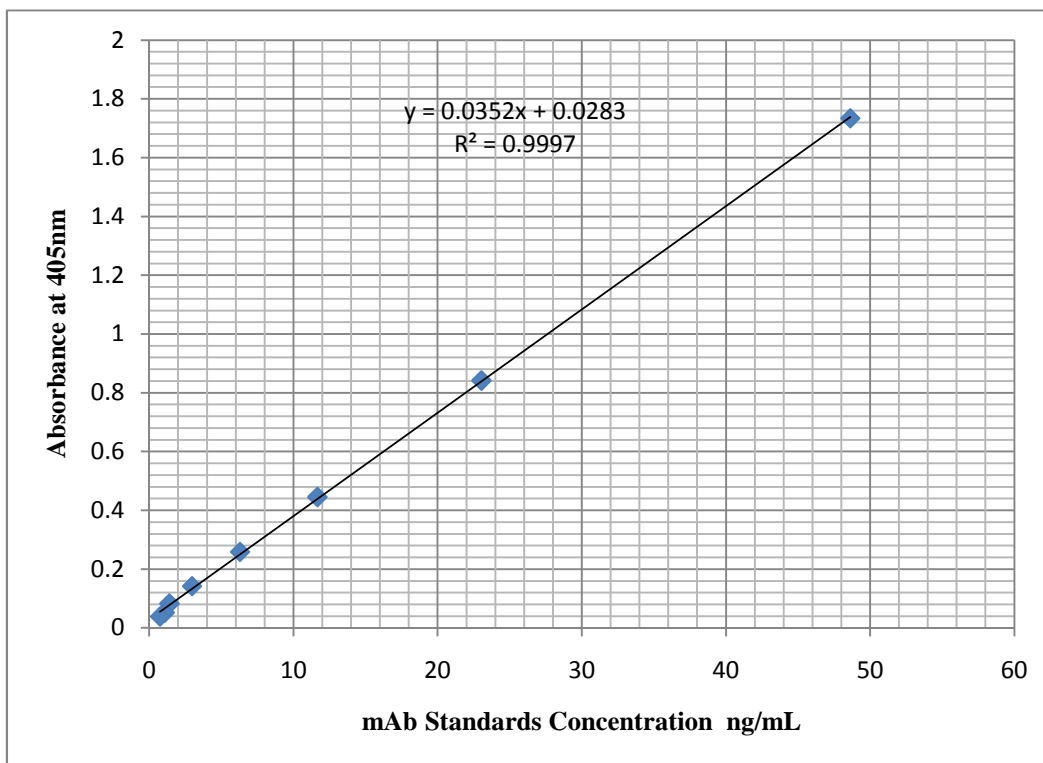


Figure 5.4: Representative standard curve for IgG quantification.

Samples were assayed in parallel in double serial dilution. A dilution in the linear range figure was used to calculate the concentration of antibodies. Antibody concentration by *in vitro* cell culture usually ranges from 20-50 $\mu\text{g/mL}$.

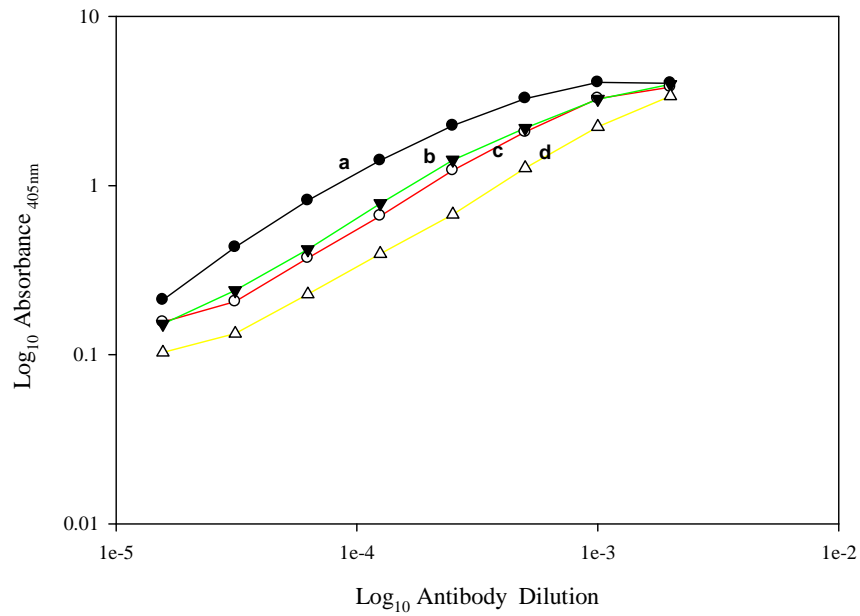


Figure 5.5: Shows the antibody OD at 405 nm in a serial dilution for use in antibody determination. Clone mAbs: a) 2E5, b) 10C2, c) 29E5 and d) 6C4.

Absorbances within the linear range were used in the calculation of antibody concentration. See Table 5.4 for full list antibody concentrations for all the clones.

Table 5.5: Summary of isotyping and antibody concentration

Mice	Clone	2,4 TDI- HSA	2,6 TDI- HSA	IgG1	IgG2 _a	IgG2 _b	IgG3	IgM	Concentration µg/mL
2,4 TDI-KLH	2E5	✓		✓					39
	10C2	✓		✓					101
	40C6	✓	✓		✓				21
	41B9	✓		✓					95
	42E2	✓	✓	✓					52
	43B4	✓	✓	✓					169
	43G6	✓	✓	✓					65
	46G1 0	✓	✓			✓			27
	49B1 0	✓	✓	✓					70
	50B5	✓	✓	✓					23
	50F8	✓	✓	✓					100
	51E6	✓	✓	✓					48
	52G1 1	✓	✓	✓					40
	56G8	✓			✓				16
	57B5	✓	✓	✓					59
	57D5	✓	✓		✓				11
	57F2	✓		✓					37
	59E5	✓	✓	✓					91
	59B3	✓		✓					31
	60D1 0	✓	✓		✓				23
	60G2	✓	✓	✓					104
	61C2	✓	✓		✓				33
	62E4	✓	✓	✓					52
	62G5	✓	✓		✓				17
	63D3	✓	✓	✓					29
	66C2	✓	✓	✓					51
	66F7	✓	✓	✓					60
	66F1 0	✓	✓	✓					32
	67C4	✓			✓				32
	73F1 1	✓	✓		✓				46
	75C8	✓	✓	✓					75
	75E4	✓	✓	✓					74
	77E6	✓	✓	✓					80
	79C7	✓			✓				35
	79G3	✓	✓			✓			39
	79G7	✓	✓		✓				33

Mice	Clone	2,4 TDI- HSA	2,6 TDI- HSA	IgG1	IgG2 _a	IgG2 _b	IgG3	IgM	Concentration µg/mL
2,6 TDI-KLH	16C6	✓	✓	✓					56
	31F2	✓	✓		✓				20
	32B6	✓	✓	✓✓					71
	53C2	✓	✓		✓				34
	53C6	✓	✓	✓					88
	54F8	✓	✓			✓			85
	57G8	✓	✓			✓			66
	59E5		✓				✓		6
	60C5	✓	✓	✓					10
	60C1 1	✓	✓	✓					70
	68D3	✓	✓				✓		38
	68E4	✓	✓		✓				134
	68D5	✓	✓		✓				130
TDI Vapor Exposed	6C4	✓	✓					✓	0.129
	16F4	✓	✓					✓	0.164
	27G6	✓	✓					✓	0.276
	29E6	✓	✓					✓	0.160
	35D6	✓	✓					✓	0.82
	40C6	✓	✓					✓	0.394
	41G6	✓	✓					✓	0.240
	42C3	✓	✓					✓	0.260
	56F9	✓	✓					✓	0.467

The species and isotype of each mAb must be determined for assay format development and future purification. IgM antibodies are useful for cytotoxicity assays but do not bind to protein A¹¹⁴. There are several methods available for isotype determination and all involve use of isotype-specific secondary anti-IgG antibodies combined with a visual detection system.

Table 5.6 : Comparison of antibody-binding characteristics for Protein A and Protein G¹¹⁵ that are commonly used for mAb purification

	Isotype	Protein A	Protein G
Mouse	IgG ₁	W	M
	IgG _{2a}	S	S
	IgG _{2b}	S	S
	IgG ₃	S	S
	Total IgG	S	S
	IgM	NB	NB
Rat	IgG ₁	W	M
	IgG _{2a}	NB	W
	IgG _{2b}	NB	W
	IgG _{2c}	S	S
	Total IgG	W	M

Legend: W= weak binding NB=no binding M= medium binding S= Strong

Supernatant fluids from standard flask cultures usually range from 10 to 50 ug/mL¹¹⁴, although this can vary according to the individual hybridoma and the degree of confluence of the cultures at the time of harvesting.

CHAPTER 6

CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST TOLUENE DIISOCYANATE HAPTENATED PROTEINS FROM VAPOR- EXPOSED MICE

6.1 Introduction

TDI is an industrially important polymer cross-linker used in the production of polyurethane. Workplace exposure to TDI and other diisocyanates is reported to be a leading cause of low molecular weight-induced OA. Currently we have a limited understanding of the pathogenesis of OA. mAbs that recognize TDI bound proteins would be valuable tools or reagents, both in exposure monitoring and in TDI-induced asthma research. In this chapter, we sought to develop toluene TDI-specific mAbs for potential use in the development of standardized immunoassays for exposure and biomarker assessments. Mice were exposed 4 h/day for 12 consecutive weekdays to 50 ppb, 2,4;2,6 TDI vapor (80=20 mixture). Splenocytes were isolated 24 h after the last exposure for hybridoma production. Hybridomas were screened in a solid-phase indirect enzyme-linked immunosorbent assay against a 2,4 TDI-human serum albumin (2,4 TDI-HSA) protein conjugate. A total of 10 hybridomas were obtained and subsequent isotyping showed that they all secreted IgM mAbs. All hybridomas produced κ light chain antibodies and their concentrations in the culture supernat fluid ranged from 82 to 467 ng/mL. Although most mAbs strongly reacted with dNCO-HSA conjugates, they also showed significant cross-reactivity with

unconjugated HSA (Appendix B). Only three mAbs designated as 16F4, 29E5 and 56F9 were found to preferentially react with dNCO conjugated HSA and were selected for further characterization (Table 6.1). The properties of these MABs (isotype and reactivity to various protein-isocyanate conjugate epitopes) were characterized using ELISA, dot blot, and Western blot analyses. Western blot analyses demonstrated that some TDI conjugates form inter- and intra-molecular links, resulting in multimers and a change in the electrophoretic mobility of the conjugate. These antibodies may be useful tools for the isolation of endogenous diisocyanate-modified proteins after natural or experimental exposures and for characterization of the toxicity of specific dNCOs.

6.2 Reactivity Studies

ELISA

Preliminary ELISAs were performed to optimize the antigen concentration and to select the linear range of the mAb reactivity against 2,4 TDI-HSA. Based on the reactivity shown in Figure 6.1, an antigen concentration of 5 µg/mL was selected for the analysis of all three mAbs.

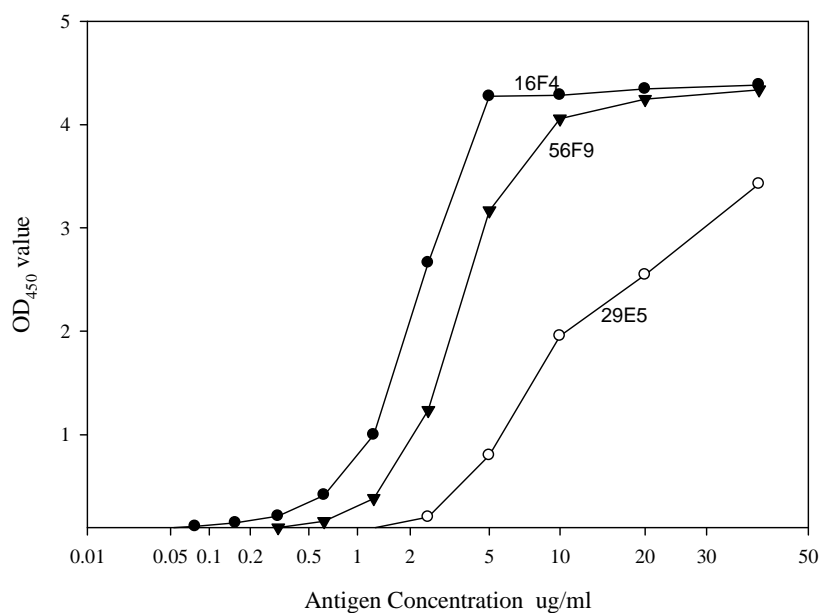


Figure 6.1: Optimization of coating concentration of 2,4 TDI-HSA. Each value represents the mean of duplicate wells. The mean control OD₄₀₅ was 0.02, for HSA.

Each antibody was titrated as shown in Figure 6.2 and mAbs 16F4, 29E5 and 56F9 were used at a concentration of 10 ng/mL, 80 ng/mL and 30 ng/mL, respectively to ensure an antibody concentration in the linear range of reactivity.

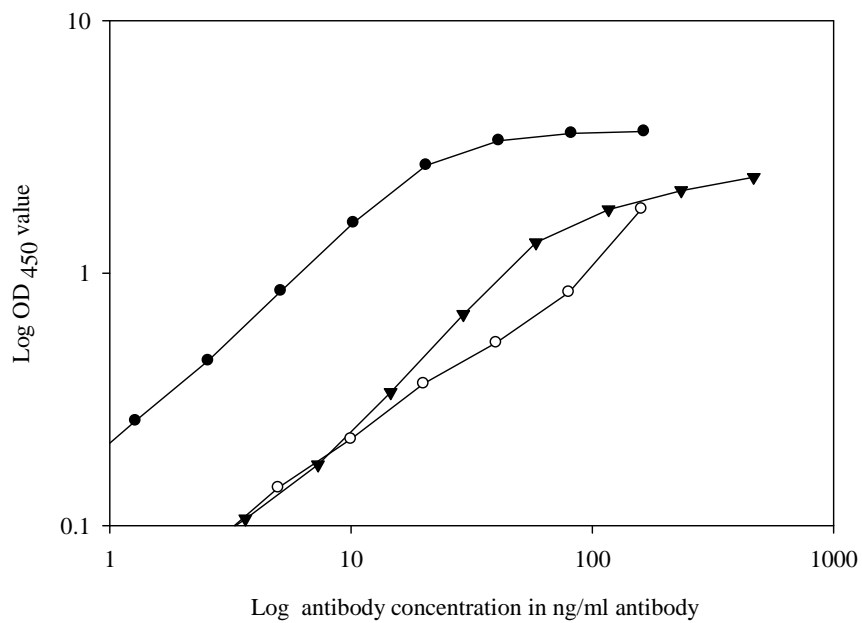


Figure 6.2: Representative Antibody Titration. mAb titration was performed to identify the mAb concentration to be used in subsequent reactivity studies. Log-linear responses were found for mAb up to 100 ng/mL under the present ELISA conditions.

Table 6.1: Summary of reactivity for mAbs 16F4, 29E5 and 56F9.

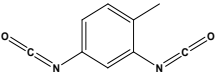
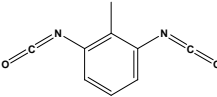
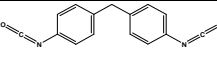
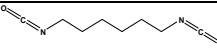
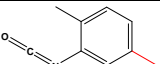
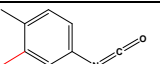
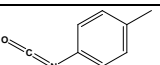


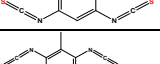
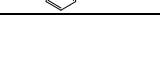
Chemical Name	Structure	Test Antigen (5 µg/mL)	Optical density [405 nm, 30 min]		
			16F4 (10 ng/mL)	29E5 (80 ng/mL)	56F9 (29 ng/mL)
Human Serum Albumin		HSA	0.078±0.047	0.065±0.004	0.089±0.022
2,4-toluene diisocyanate		2,4-TDI-HSA	2.512±0.121	1.274±0.128	2.140±0.109
		2,4-TDI-KLH	0.578±0.037	3.635±0.164	0.881±0.088
		2,4-TDI-MSA	0.398±0.071	0.236±0.067	0.443±0.043
		2,4-TDI-keratin	-	-	-
		2,4-TDI-lysozyme	0.012±0.005	0.004±0.004	0.031±0.015
		2,4-TDI-collagen	-0.019±0.01	0.022±0.005	0.011±0.005
		2,4-TDI-collagen	-	-	-
2,6-toluene diisocyanate		2,6-TDI-HSA	2.356±0.039	1.824±0.180	1.596±0.02
		2,6-TDI-KLH	0.047±0.028	0.267±0.013	0.142±0.02
		2,6-TDI-MSA	0.279±0.027	0.092±0.028	0.367±0.066
		2,6-TDI-keratin	-	-	-
		2,6-TDI-lysozyme	0.002±0.005	0.016±0.011	0.039±0.011
		2,6-TDI-lysozyme	0.008±0.002	0.013±0.007	0.012±0.012
		2,4 TDI-collagen	-	-	-
2,4;2,6 TDI (Industrial mix)		2,4;2,6 TDI-HSA	0.723±0.038	0.954±0.08	0.149±0.036
4,4-methylene diphenyl diisocyanate		MDI-HSA	0.491±0.029	0.370±0.017	0.091±0.017
hexamethylene diisocyanate		HDI-HSA	0.858±0.023	1.590±0.132	0.765±0.022
2,5-dimethyl phenylisocyanate		2,5-DMPI-HSA	0.832±0.097	0.150±0.046	0.174±0.028
3,4-dimethyl phenylisocyanate		3,4-DMPI-HSA	0.633±0.03	0.132±0.015	0.226±0.032
4-toluene isocyanate		PTI-HSA	0.015±0.021	0.025±0.191	0.020±0.091
2-toluene isocyanate		OTI-HSA	0.070±0.037	0.009±0.015	0.087±0.01
phenyl isocyanate		PI-HSA	-	0.011±0.012	0.007±0.006
2,4-toluene diisothiocyanate		2,4-TITC-HSA	0.096±0.018	0.119±0.009	0.227±0.017
2,6-toluene diisothiocyanate		2,6-TITC-HSA	0.006±0.041	-	0.135±0.05

Table 6.1 provides a summary of the reactivity of the mAbs toward proteins conjugated with dNCOs, NCOs and NCSs. It can be seen that all three mAbs react with 2,4 TDI-HSA, 2,6 TDI-HSA, HDI-HSA, 2,4 TDI-MSA and 2,4 TDI-KLH. Monoclonals 16F4 and 29E5 also react with 2,4;2,6 TDI-HSA conjugates and MDI – HSA. MAbs 16F4 and 59F9 also react with 2,6 TDI-MSA. 16F4 was the only mAb to react with 2,3 DMPI and 2,5 DMPI while 29E5 was the only mAb to react with 2,6 TDI-KLH. None of the other conjugates were recognized by the mAbs in the ELISA format.

Western blot Analyses.

The representative Western blot analysis for mAb 56F9 (Figure 6.3) showed that it reacted with the denatured form TDI-conjugated proteins. The pattern of reactivity of all 3 mAbs was similar; varying only in intensity of the reaction.

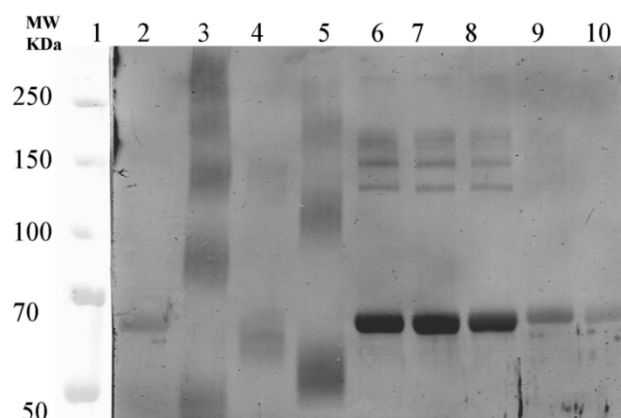


Figure 6.3: Western Blot Analyses of mAb 56F9. Lane 1, Precision Plus Protein™ standard (Biorad); lane 2, HSA; lane 3, 2,4 TDI-HSA; lane 4, 2,6 TDI-HSA; lane 5, 2,4:2,6 TDI-HSA; lane 6, 2,3 DMPI-HSA; lane 7, 2,5 DMPI-HSA; lane 8, 3,4 DMPI-HSA; lane 9, OTI-HSA and lane 10, PTI-HSA

The mAbs reacted to TDI conjugated proteins that contained both intra- and intermolecular TDI mediated cross-links. There was also some reactivity in the Western blots that was not seen in the ELISA assay format. All mAbs reacted with methyl substituted monoisocyanates in Western blots, yet only 16F4 was reactive in ELISA (see Table 6.1). The OTI- and PTI-HSA conjugates had greater reactivity in Western blots than ELISA for all three mAbs.

Dot Blot Analysis.

Figure 6.4 A-C are dot blots that show mAb 16F4, 29E5 and 56F9, reactivity to native and denatured protein conjugates, A and B respectively. All the mAbs had similar reactivity in the denatured protein blots, which only differed in intensity. 16F4 and 56F9 had similar reactivities towards both native forms of isocyanate-protein conjugates, while 29E5 reacted towards 2,4 TDI/KLH and 2,6 TDI-KLH.

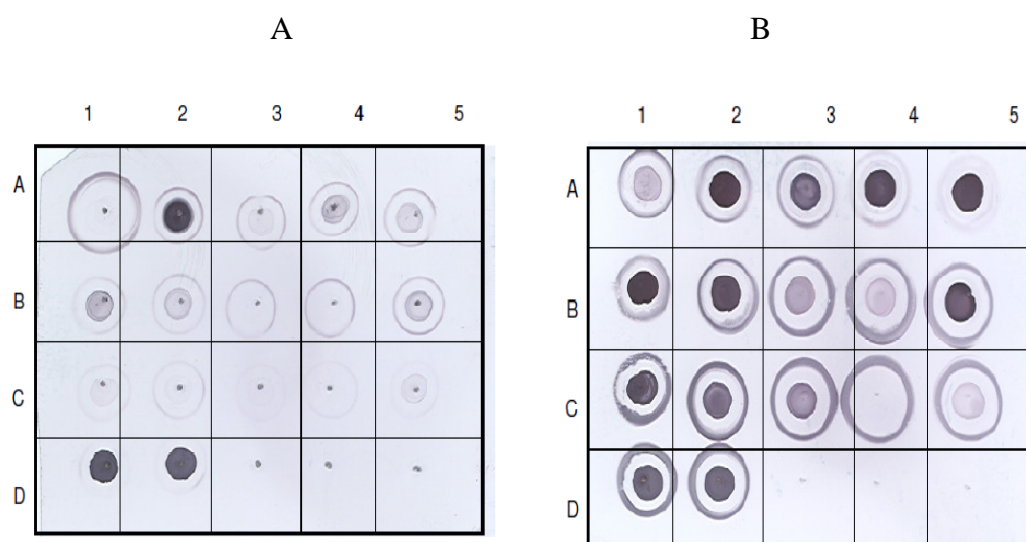


Figure 6.4A: Dot ELISA for 16F4 with (A) native and (B) denatured protein.
N.B: Key below Figure 6.4C.

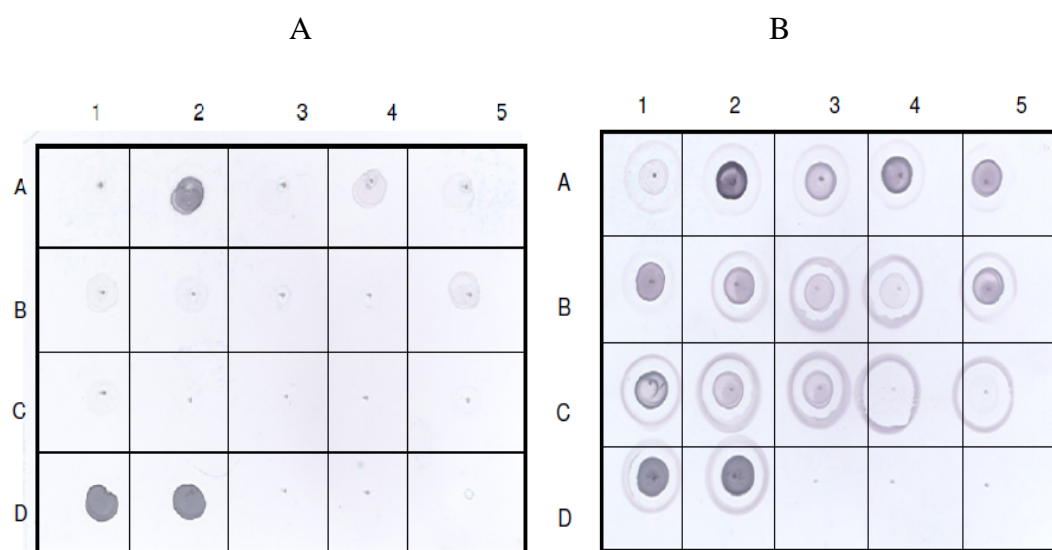


Figure 6.4B: Dot ELISA for 29E5 with (A) native and (B) denatured protein

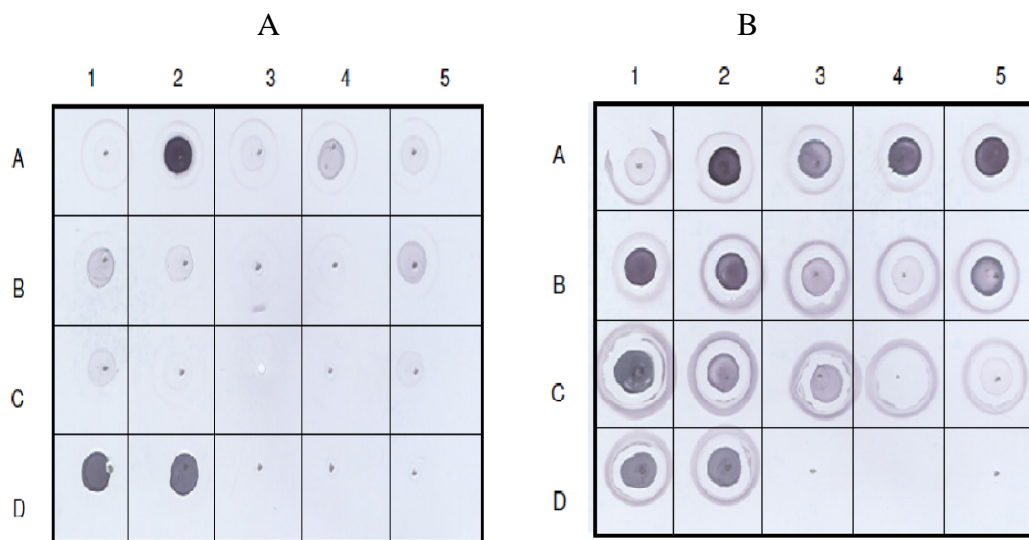


Figure 6.4C: Dot ELISA for 56F9 with (A) native and (B) denatured protein

Key: For Figures 6.4 A, B and C

Lane A1: HSA	Lane A2: 2,4 TDI-HSA	Lane A3: 2,6 TDI-HSA
Lane A4: HSA-2,4;2,6 TDI (industrial mix)	Lane A5: HSA-2,5 DMPI	
Lane B1: HSA-3,4 DMPI	Lane B2: HSA-2,4 DMPI	Lane B3: HSA-OTI
Lane B4: HSA-OTI	Lane B5: HSA-MDI	Lane C1: HSA-HDI
Lane C2: HSA-2,4TITC	Lane C3: HSA-2,6 TITC	Lane C4: HSA-PI
Lane C5: KLH	Lane D1: KLH-2,4 TDI	Lane D2: KLH-2,6 TDI
Lane D3-D5: Empty		

All mAbs showed strongest reactivity towards 2,4 TDI-HSA, 2,4 TDI-KLH and 2,6 TDI-KLH and reacted least with 2,4;2,6 TDI-HSA (mix). 29E5 had no other unique reactivities while mAbs, 16F4 and 56F9 also reacted with 2,6 TDI-HSA, methyl substituted monoisocyanates, HDI-HSA and MDI-HSA. The pattern of reactivity of the mAbs to denatured conjugated proteins observed in the dot blots (Figure 6.4) was

consistent with that observed by Western blot analyses. None of the mAbs reacted with HSA native protein.

6.3 Discussion

All mAbs produced herein reacted with proteins that contained intra- and inter-molecular TDI-mediated cross-linking, suggesting that their reactivity was not impaired by the presence of cross-linking. Examination of the mAbs reactivity towards monoisocyanate-haptenated proteins was used to assess the need of molecular cross-linking on the ability of the mAbs to recognize TDI-haptenated proteins. OTI and PTI are monoisocyanates with the second isocyanate group removed from the ortho and para positions relative the methyl group. 2, 3 DMPI, 2,5 DMPI and 3,5 DMPI are monoisocyanates with the ortho and para positions substituted with methyl groups. PI has only one isocyanate and no methyl group attached to the phenyl ring. All monoclonal antibodies recognized monoisocyanate haptenated albumin suggesting that the cross-linking was not critical to immununogen recognition.

The assay format, however, was found to influence the mAb reactivity with the various haptenated species. Having an isocyanate or a substitution at the ortho position on the tolyl group was critical to mAb recognition in the ELISA format. This was true for all three mAbs, suggesting a possible influence of ELISA plate chemistry in the interactions between the mAbs and haptenized protein. The mAbs recognized haptenated albumin in which there was substitution of one isocyanate on the tolyl

group, (in the Western blot and Dot blot formats) but not simple removal of the isocyanate. Interestingly, higher molecular weight proteins than ~66.5 KDa in the stock HSA as observed in the protein blot were also haptenated by TDI and recognized by the mAbs (Figures 3.2 and 6.2). These mAbs also recognized MDI and/or HDI conjugated HSA, in addition to the TDI-HSA. This suggests that the resultant bond formation following reaction of the dNCO to protein was important in recognition of TDI haptenated proteins by the mAbs.

The reactivity of the antibodies may also be influenced by choice of the experimental carrier protein. mAbs reacted with 2,4 TDI bound to HSA or MSA and also to KLH which is a non mammalian protein indicating the importance of the dNCO- protein bond rather than the source species of the carrier protein. Although lysozyme and keratin were demonstrated by matrix assisted laser desorption ionization and/or loss of primary amines (Fig 3.3) to be haptenated, none of the mAbs in present study showed reactivity towards their conjugates. This may be due to the poor binding of the conjugates to ELISA plate surface, lower haptenation rates or steric inaccessibility of the TDI group on these carrier proteins.

A total of 10 mAbs (all IgMs) generated from TDI vapor-exposed mice were isolated during the initial screening against TDI-HSA. Only 3 mAbs, however, showed none or very low reactivity to unconjugated HSA (see Appendix B). The reason for the high reactivity of the other mAbs with HSA is not known but we were unable to block this

binding with either milk or BSA. It is possible that haptination of proteins may also lead to recognition of the self protein by the immune system as seen with exposure to aldehydes ¹¹⁶. This, however, has not been investigated for isocyanate exposures. Interestingly, these TDI vapor sensitized mice had IgG anti-2,4 TDI-MSA titers of $18,106 \pm 7994$ (sera dilution to reach an ELISA OD >0.1). IgM anti-2,4 TDI-MSA was not assessed in these mice; however, anti-2,4 TDI-MSA IgM was not observed in the sera of another group of mice exposed to an identical inhalation protocol .

Future potential applications of these mAbs include the isolation of endogenous carrier proteins that are conjugated following natural or experimental exposure episodes. The purified proteins can then be identified and their structure and chemical linkages determined by mass spectrometry. In chapter 4 we demonstrated the potential for such methods for isocyanate-peptide adducts using tandem mass spectrometry ¹¹⁷. The mAbs may also be useful reagents for the development of immunoassays designed for dNCO exposure assessments.

In summary, we have successfully developed monoclonal antibodies that recognize dNCO–protein adducts from TDI vapor-exposed mice. The specificity of these antibodies was demonstrated by ELISA, Western blot and Dot blot analyses. The antibodies may be useful tools for the identification of endogenous dNCO-modified proteins and the characterization of the importance of the type of the chemical linkage in terms of isocyanate toxicity.

CHAPTER 7

CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST 2,4/2,6 TDI-HSA FROM 2,4/2,6 TDI-KLH IMMUNIZED MICE

7.1 Introduction

Diisocyanates (dNCOs) are very reactive low molecular weight chemicals that are widely used in the manufacture of polyurethane products. Diisocyanate exposure is one of the most commonly reported causes of occupational asthma. Although dNCOs have been identified as causative agents of respiratory diseases, the specific mechanisms by which these diseases occur are largely unknown. Toluene diisocyanate (TDI)-specific monoclonal antibodies (mAb) with potential use in immunoassays for exposure and biomarker assessments were produced in the present study. A total of 49 unique mAbs were produced (29 IgG₁, 14 IgG_{2a}, 4 IgG_{2b} and 2 IgG₃) against 2,4- and 2,6-toluene diisocyanate (TDI) bound protein. The reactivities of the mAbs were characterized by ELISA, Dot blot and Western blot against various mono- and diisocyanate and dithioisocyanate protein conjugates. A subset of the mAbs were specific for 2,4 or 2,6 TDI-conjugated proteins only, while others reacted to multiple dNCO conjugates including methylene diphenyl diisocyanate (MDI) - and hexamethelene diisocyanate (HDI) – human serum albumin (HSA). In general, 2,4/2,6 TDI reactive mAbs displayed (1) stronger recognition of mono-NCO haptenated proteins when the NCO was in the ortho position relative to the tolyl group, and were able to discriminate between (2) isocyanate and isothiocyanate conjugates (i.e.

between the urea and thiourea linkage); and (3) between aromatic and aliphatic dNCO. The mAbs produced were not carrier protein specific with estimated affinity constants toward TDI-HSA ranging from 2.21×10^7 to $1.07 \times 10^{10} \text{ M}^{-1}$. Preliminary studies using TDI vapor exposed cells suggest potential utility of these mAbs for both research and biomonitoring.

7.2 Results

Antibody concentrations in the culture supernatant fluids range from 0.160 $\mu\text{g/mL}$ to 169 $\mu\text{g/mL}$ with an average concentration of 52 $\mu\text{g/mL}$. Isotyping showed 29 hybridomas secreted IgG₁, 14 IgG_{2a}, 4 IgG_{2b}, and 2 IgG₃ antibodies. All hybridomas produced κ light chains. Preliminary ELISAs were performed to optimize the antigen concentration and to select the linear range of the mAb reactivity against 2,4 TDI-HSA.

Monoclonal antibodies were grouped in 7 groups based on similar patterns of reactivity to the protein conjugated listed in Table 7.1. Seven mAbs react only with 2,4 TDI-HSA, and 1 mAb reacted only to 2,6 TDI-HSA. Table 7.1 shows ELISA results for 7 mAbs, each representing one group of mAbs with similar reactivity as well as the dot ELISA results for mAbs tested by dot ELISA. The 10C2 group only reacted with 2,4 TDI-conjugated proteins while 59E5 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-MSA. None of the mAbs reacted with 2,4/2,6 TDI-Lysozyme (data not

shown). The 60G2 and 79G6 groups reacted with HDI-HSA and MDI-HSA conjugates. See Appendix C; Table 1 and 2 for detailed reactivity of 2,4/2,6 TDI-KLH immunized mice) The mAbs displayed similar reactivities in the Dot blot assays (Figure 7.1) to ELISA results. There were no qualitative differences between the native and denatured dot blots, although denatured proteins seemed to result in higher assay sensitivity. The mAbs were also tested against a commercial 80/20 2,4/2,6 TDI mixture and MDI containing Gorilla glue (The Gorilla Glue Company, Cincinnati, OH) conjugated to HSA. All mAbs reacted with the commercial 80/20 2,4/2,6 TDI mixture. Interestingly, 60G2 reacted with the Gorilla glue conjugate in the ELISA format but not in the dot blots.

Table 7.1: Results of the ELISA analysis of 7 monoclonal antibodies against toluene diisocyanate conjugated proteins^a.

Test (4µg/ml)	Antigen	ELISA readings (OD _{405nm}) and Dot blot scoring (+++)						
		2E5 (8)*	60G2 (3)	62G5 (4)	79G7 (1)	16C6 (3)	32B6 (5)	59E9 (1)
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
2.4 TDI-keratin		1.08	3.11	0.55	1.31	0	2.01	0
		++	+++	++	+++	0	+	0
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
HDI-HSA		0	0.21	0	0.75	0	0	0

Test (4µg/ml)	Antigen	ELISA readings (OD _{405nm}) and Dot blot scoring (+++)						
		2E5 (8)*	60G2 (3)	62G5 (4)	79G7 (1)	16C6 (3)	32B6 (5)	59E9 (1)
		0	+	0	+	0	0	0
MDI-HSA		0	0.66	0	0.21	0	0	0
		0	++	0	+	0	0	0
2,4;2,6 TDI-HSA		3.85	3.81	3.26	3.56	3.78	2.80	1.13
		+++	+++	+++	+++	+++	+++	+
2,5-DMPI-HSA		0	0.64	2.15	3.2	0	0.62	0
		0	+	+++	+++	0	+	0
3,4-DMPI-HSA			0.27	0.77	0.61	0	0	0
		0	+	+++	+++	0	+	0
		0	0	0	0	0	0	0
PI-HSA		0	+	0	0	0	0	0
2,4-TITC-HSA		0	0	0	0	0	0	0
		0	0	0	0	0	0	0
2,6-TITC-HSA		0	0	0	0	0	0	0
		0	0	0	0	0	0	0
OTI-HSA		0	0	3.65	3.8	0	0	0
		0	0	+++	++	0	0	0
PTI-HSA				0	0	0	0	0
		0	0	0	0	0	0	0

^aThese results represent the mean OD₄₀₅ of 4 ELISA well repeats which were corrected by subtracting the average OD 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 3 times the OD₄₀₅ value of HSA or

conjugating protein like MSA. A zero value indicates that the OD₄₀₅ or visual dot were insignificant. 2E5, 60G2, 62G5 and 76G7 are from 2,4 TDI-KLH immunized mice, while 16C6, 32B6 and 59E5 are from 2,6 TDI-KLH immunized mice. 2,4/2,6 lysozyme, 2,4 TDI-collagen, PI-HSA, PTI-HSA, and 2,4/2,6 TITC (data not shown as they had insignificant reactivities).

*Indicates number of antibodies in that group.

Dot Blot Key: +++ Strong reaction, ++ Moderate reaction, + Weak reaction

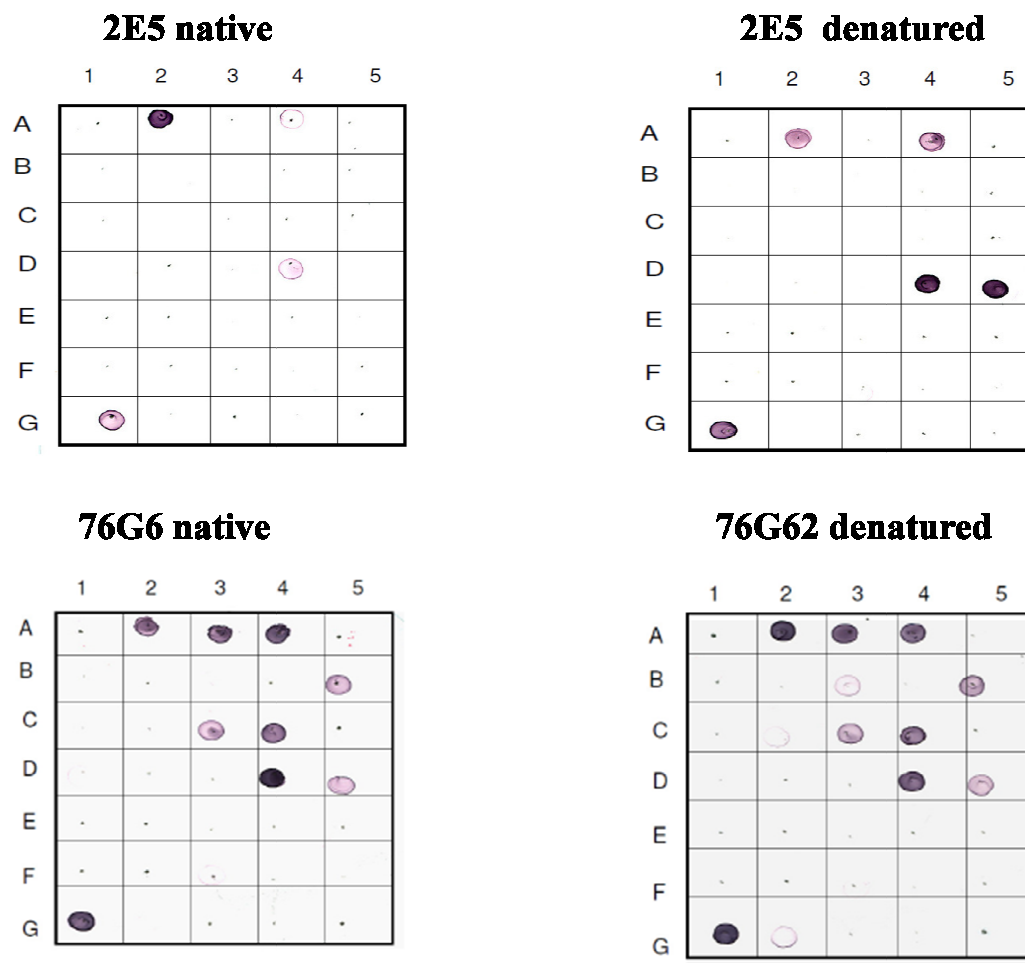


Figure 7.1: Native and Denatured Protein Dot Blot analysis of mAbs 2E5 and 76G6. Lane configuration is the same for both panels.

Key:

Lane A1: HSA	Lane A2: HSA-2,4 TDI	Lane A3: HSA-2,6 TDI
Lane A4: HSA-2,4;2,6 TDI	Lane A5: HSA-HDI	Lane B1: HSA-MDI
Lane B2: HSA-Gorilla glue	Lane B3: HSA-2,4 TITC	Lane B4: HSA-2,6 TITC
Lane B5: HSA-OTI	Lane C1: HSA-PTI	Lane C2: HSA-PI
Lane C3: HSA-3,5 DMPI	Lane C4: HSA-2,5 DMPI	Lane C5: AHSA
Lane D1: AHSA-2,4 TDI	Lane D2: AHSA-2,6 TDI	Lane D3: KLH
Lane D4: KLH-2,4 TDI	Lane D5: KLH-2,6 TDI	Lane E1: Lysozyme
Lane E2: Lysozyme-2,4 TDI	Lane E3: Lysozyme-2,6 TDI	Lane E4: Collagen
Lane E5: Collagen-2,4 TDI	Lane F1: Collagen 2,6 TD	Lane F2: Keratin
Lane F3: Keratin-2,4 TDI	Lane F4: Keratin-2,6 TDI	Lane F5: MSA
Lane G1: MSA-2,4 TDI	Lane G2: MSA-2,6 TDI	Lane G3: CH3NH2
Lane G4: CH3NH ₂ -2,4 TDI	Lane G5: CH3NH ₂ -2,6 TDI	

The reactivities of select mAbs were also analyzed using Western blot. As shown in Figure 7.2, 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/MDI bound HSA probably through specific recognition of the urea bond linkage formed by reacting NCO to the protein amine.

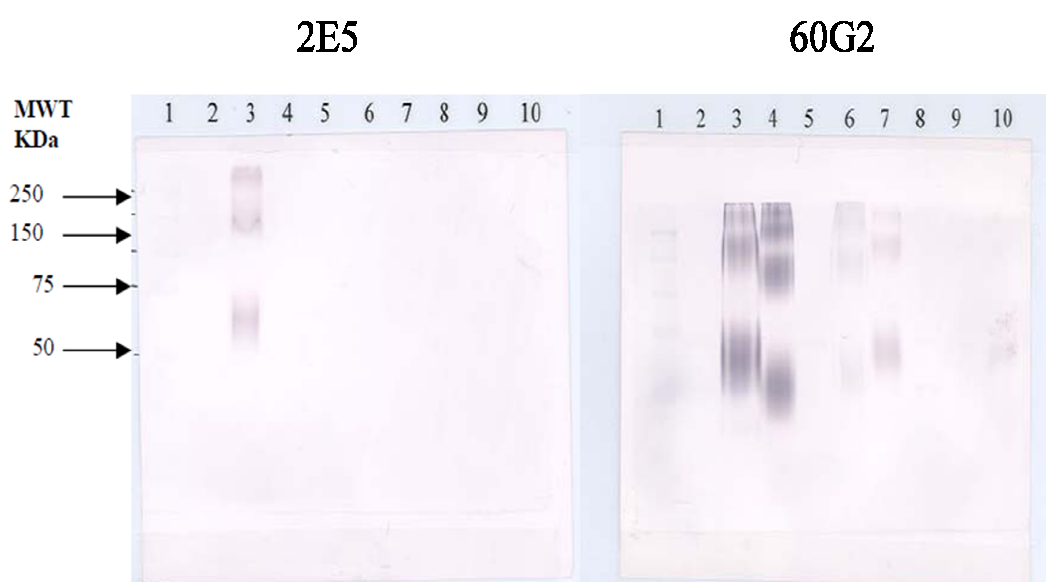


Figure 7.2: Western blots of mAb, 2E5 reactivity and mAb 60G2 reactivity.

Lane 1 MWt markers, lane 2 HSA, lane 3 HSA-2,4TDI, lane 4 2,6 TDI-HSA, lane 5 HSA-PI, lane 6 HSA-MDI, lane 7 HDI-HSA, lane 8 OTI-HSA, lane 9 HSA-PTI and lane 10 HSA-2,4 TITC

Determination of affinity constant (K_a) by ELISA

Affinity is a quantitative measure of the strength of an interaction between two independent molecules. The affinities of select antibodies were determined by ELISA using quadruple serial concentrations of antigen and constant mAb concentrations. Representative curves for mAbs 10C2 and 16C6 are shown in Figure 7.3 and the correspondingly calculated average K_a for select mAbs is shown in Table 7.2, mAb 79G7 had the lowest K_a value of $2.21 \times 10^7 \text{ M}^{-1}$ and 59E5 had the highest K_a value of $1.07 \times 10^{10} \text{ M}^{-1}$.

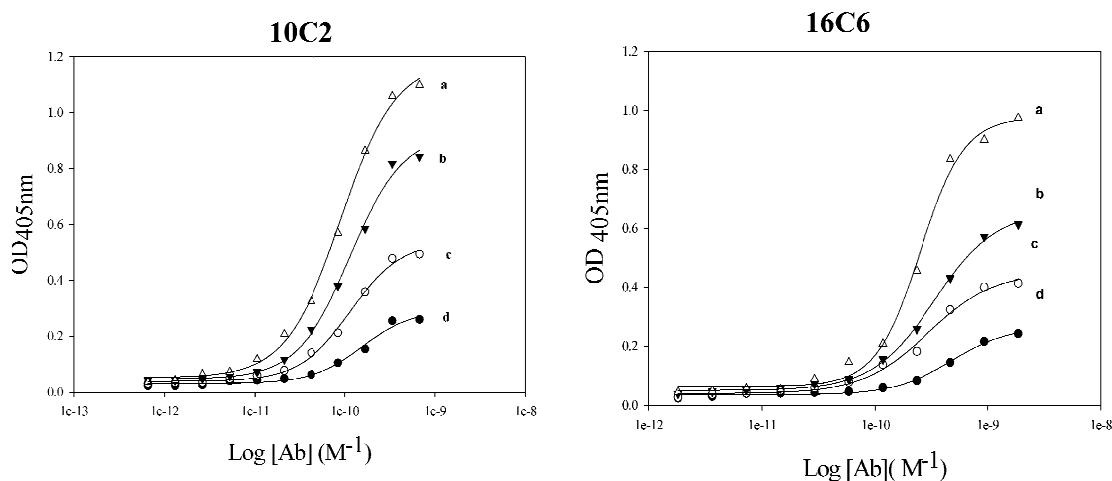


Figure 7.3: Representative binding curves from 10C2 and 16C6 employed for extrapolating the affinity constant of the mAbs. a) 2.5 $\mu\text{g/ml}$, b) 1.25 $\mu\text{g/ml}$, c) 0.625 $\mu\text{g/ml}$ and d) 0.312 $\mu\text{g/ml}$ and the antibody concentration kept constant at $6.733 \times 10^{-10} \text{ M}$ and $1.866 \times 10^{-9} \text{ M}$, respectively

Table 7.2: Affinity constant of representative mAbs against 2,4/2,6 TDI-HSA conjugates by ELISA.

2,4 TDI-HSA			2,6 TDI-HSA		
Clone	Class and subclass	Affinity constant (K_a) M^{-1}	Clone	Class and subclass	Affinity constant (K_a) M^{-1}
10C2	IgG ₁	8.80×10^9	16C6	IgG ₁	2.33×10^9
60G2	IgG ₁	2.65×10^9	31F2	IgG _{2a}	3.81×10^{10}
62G5	IgG _{2a}	2.23×10^9	32B6	IgG ₁	5.69×10^9
79G7	IgG _{2a}	2.21×10^9	53C2	IgG ₁	2.62×10^8
42E2	IgG ₁	9.73×10^8	54F8	IgG _{2b}	3.51×10^9
43B4	IgG ₁	2.9×10^7	59E5	IgG ₃	1.07×10^{10}
49B10	IgG ₁	2.49×10^7	60C11	IgG ₁	2.26×10^9
59E9	IgG ₁	2.21×10^9	68D5	IgG _{2a}	9.82×10^9

Experimental TDI Cell exposure

The overarching goal in developing IgG mAbs specific for isocyanate conjugated proteins is to develop tools and assays useful for exposure assessment, disease surveillance/diagnosis and disease research. To this end, we have tested the potential of several of our mAbs to recognize haptenized proteins formed under similar conditions that may be found in the workplace. We exposed protein solutions and live air-liquid interface cell cultures to an occupationally relevant concentration of TDI vapor (50 ppb; 80/20 2,4-/2,6-TDI). Immunoblot analysis of the MSA protein solutions demonstrated two prominent bands at 60-80 kda (TDI-MSA monomers) and

160-250 kda (TDI-MSA polymers). Solutions of MSA that were left open in the TDI atmosphere (lanes 6 and 7, Figure 7.4) showed time dependent conjugation through passive diffusion. We also examined the effect of actively drawing the TDI atmosphere over the surface of the MSA solution using the Vitrocell *in vitro* exposure system. Active flow exposure resulted in a flow rate-dependent conjugation of MSA. Conjugation of MSA was barely detectable at a flow rate of 20 ml/min (lane 4, Figure 7.4) whereas marked conjugation was observed at 200 ml/min (lane 5, Figure 7.4) for 1 hour. Next, we determined if vapor TDI (50 ppb) could conjugate cell-associated proteins using live A549 cells at the air-liquid interface. These cultures were left open to the TDI atmosphere to facilitate passive diffusion for a duration of 1 hour (lanes 2 and 3, Figure 7.4). Protein conjugation patterns resulting from these exposures were similar to the MSA solutions with bands concentrated around 80 kda and 250 kda. A549 cells are a human alveolar adenocarcinoma cell line and served as a tool for proof of concept.

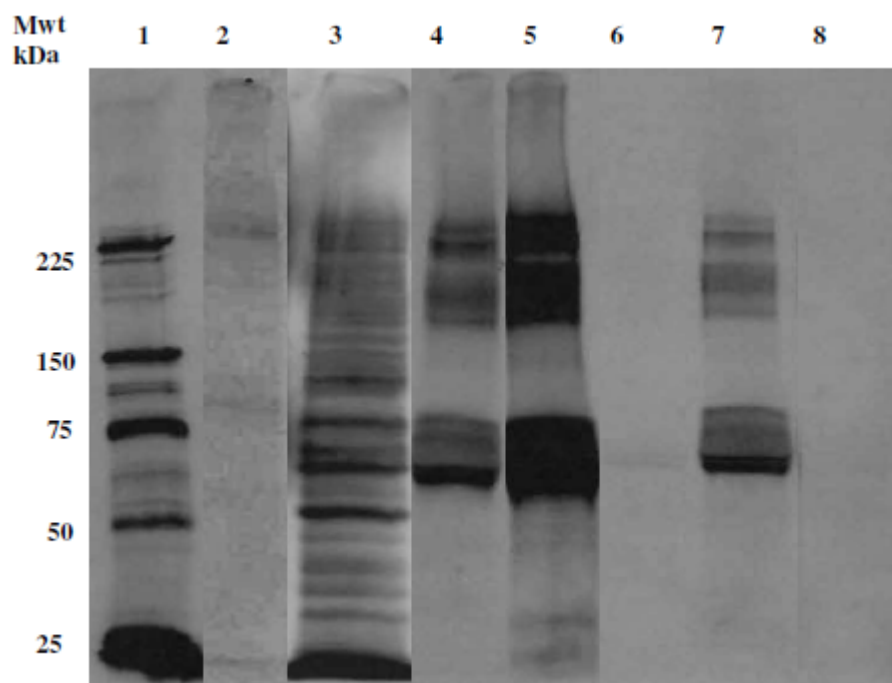


Figure 7.4: Western Blot of TDI (Vapor Exposed Mouse Albumin (MSA) and Human Lung Epithelial Cells A549 and developed with anti-TDI-protein mAb 60G2. Dark bands on MSA and cell extract lanes indicate binding of mAb. Lane 1) Pre-stained MW Marker, lane 2) A549 Cells (TDI passive diffusion, 1 hr) and 3) BEAS-2B cells (TDI passive diffusion, 4 hrs), lane 4) TDI-MSA (TDI diffusion rate 20 mL/min, 1hr) , lane 5) TDI-MSA (TDI diffusion rate 200 mL/min, 1hr), lane 6) TDI-MSA(TDI passive diffusion, 4 hrs), lane 8) Unexposed MSA(negative control) , lane 8).

Our next series of experiments employed BEAS-2B cells, a human bronchial epithelial cell line, which represents an appropriate *in vitro* to study the biological effects of TDI on airway epithelium. BEAS-2B cells were grown to confluence and actively exposed to 50 ppb TDI vapor using the Vitrocell exposure system. Flow rate across the surface of the air-liquid interface cultures was set at 100 ml/min to approximate the minute ventilation of a mouse. Immunoblot analysis shows numerous TDI-specific protein bands in the range of 30-250 kda (Figure 7.5). The binding of mAbs to TDI-conjugated BEAS-2B proteins was variable with the 2,4-TDI conjugate binding mAbs generally showing stronger reactivity .

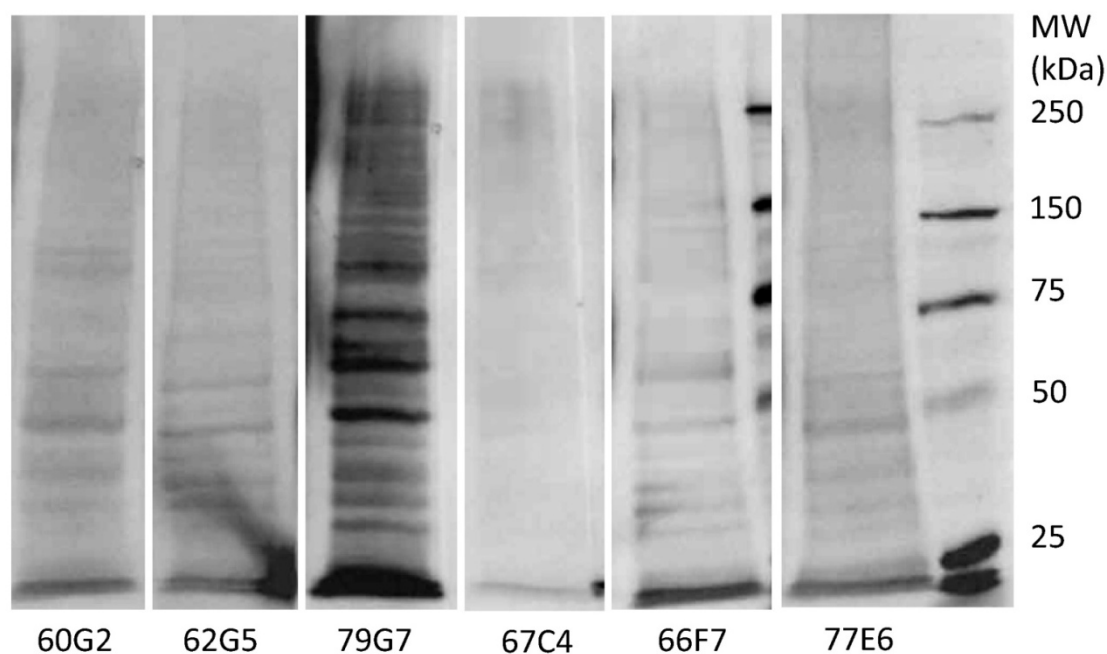


Figure 7.5 Immunoblot analysis of BEAS-2B protein extracts following exposure to 50 ppb TDI vapor for 4 hours at a flow rate of 100 ml/min.

Cell extracts separated by electrophoresis and then subjected to immune-detection using mAbs 60G2, 62G5 79G7, 67C4, 66F7 and 77E6.

7.3 Discussion

In Chapter 6 we successfully produced TDI-specific mAbs from the spleens and lymph nodes of mice with TDI rhinitis following inhalation of TDI-vapor¹¹⁷. However, all clones isolated in that study were IgM, an isotype that is not ideal for long term storage and development of assays. Therefore the objective of present study was to develop a unique set of high affinity IgG mAbs specific for isocyanate conjugates. The present approach employed TDI-conjugated KLH as the immunogen and we obtained IgG mAbs with a wide variety of specificities that should be relevant for both research and exposure characterization. The hybridomas were screened against TDI-HSA that was generated by reacting TDI to HSA at a 40:1 molar ratio. Wienswiski *et al*⁸⁸ reported that MDI-specific IgG from pooled human sera of individuals exposed to MDI displayed the greatest amount of binding to the 40:1 MDI:HSA reaction ratio. TDI quickly hydrolyzes or reacts with nucleophiles in the body, thus dNCO specific antibody production is not found. In addition, dNCOs would react directly with nucleophilic moieties on mAb preventing their use to directly detect dNCO.

The majority of mAbs that were produced cross-reacted with multiple dNCOs but mAbs specific for 2,4 TDI- or 2,6 TDI- protein were also generated (Table 7.1). In general, mAbs that recognized both 2,4 and 2,6 TDI-HSA, displayed stronger recognition to mono-NCO haptenated proteins 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 (Figure 7.5). 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 immuno-assay dependence since the Dot blots and the ELISA were highly concordant. Greater mAb reactivity was observed denatured protein conjugates in the Dot blots probably due to the exposure of cryptic TDI epitopes by unfolding the protein. This is in agreement with our earlier observation with the isocyanate-specific IgM mAb reactivity derived from TDI-vapor exposed mice¹¹⁷. Western blots also show extensive polymerization and dimerization of the dNCO-protein conjugates showing reactivity at 150 KDa and above. Antibody staining indicating molecular sizes less than HSA are most likely attributable to intramolecular TDI cross-linking preventing complete protein denaturation and faster migration through the gel. The overall reactivity pattern is summarized as below in Figure 7.6 overleaf.

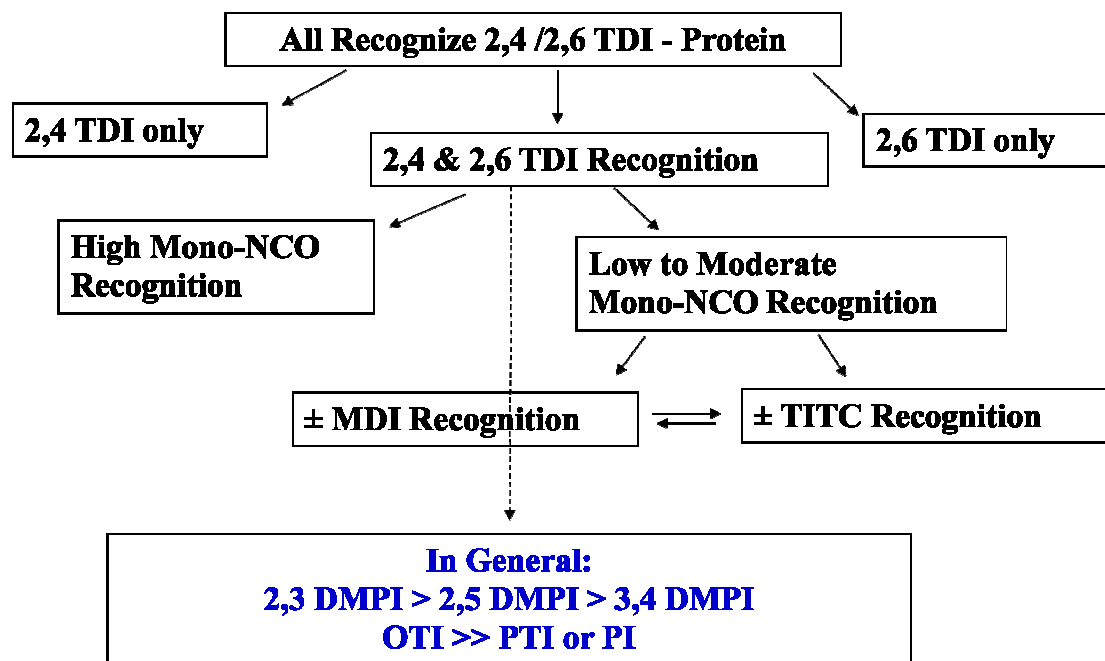


Figure 7.6. Reactivity pattern for TDI mAbs

Svensson-Elfmark and others¹¹⁸ demonstrated cross reactivity between rats repeatedly exposed to TDI exhibiting immune reactivity against methyl isocyanate-protein conjugates. Human studies have previously shown cross-reactivity between IgG antibodies of exposed workers to MDI and HSA conjugates with other isocyanates such as TDI or HDI^{55,119}.

There was little immuno-assay dependence since the Dot blots and the ELISA were in good agreement. Greater mAb reactivity was observed with denatured protein conjugates in the dot blots probably due to the exposure of cryptic TDI epitopes by unfolding the protein. This is in agreement with our earlier observation with TDI-vapor exposed IgM mAb reactivity¹¹⁷. Western blots also show extensive polymerization and dimerization of the dNCO-protein conjugates showing reactivity at

150 KDa and above. Antibody staining, indicating molecular sizes less than HSA are most likely attributable to intramolecular TDI cross-linking preventing complete protein denaturation and faster migration through the gel.

The affinity constant (K_a) of mAb is an important parameter for the application of mAbs⁷⁰. High affinity mAb increase the specificity and sensitivity of immunoassays and may be more useful in 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 the most specific reactivity to 2,6 TDI-protein (only), 59E5, also had the highest affinity, while the more promiscuous mAb, 79G7, had the lowest affinity to 2,4 TDI-HSA. As expected, the more discriminating mAbs had greater affinities than the less discriminating mAbs.

A preliminary *in vitro* TDI vapor exposure study was performed using mAb 60G2 as the detection antibody. This mAb displayed a broader range of reactivity in our characterization studies (Table 7.1). Recognition of TDI haptenated MSA was exposure-dependent with greater mAb reactivity with higher exposure flow rates or higher exposure time. The immune-blot pattern of mAb reactivity to the TDI vapor conjugated MSA was similar to the liquid infusion conjugation method used to produce the positive control for these assays suggesting that TDI may react similarly with proteins in the fluid lining the airway mucosa. Significantly, detection of TDI

conjugated proteins isolated from A549 cells exposed to only 50 ppb TDI vapor for 1 h was demonstrated by immune-blot analysis using mAbs produced in present study demonstrates the physiological relevance of the anti-TDI-protein mAbs.

We also exposed BEAS-2B cells, a human bronchial epithelial cell line, to TDI vapor for 4 hours at 100 ml/min. Immunoblot analysis was performed with several of our mAbs and demonstrates the presence of a large number of protein bands throughout the 30-250 kDa size range. These bands are likely to represent a myriad of conjugated proteins and their respective polymers and is the first step towards identification of potential epithelial protein targets following TDI exposure. The identity of the specific cellular proteins bound by TDI has not yet been demonstrated. The immune-blot for A549 and BEAS-2B demonstrates that they are not all HSA as their molecular weight range is outside that of albumin. Current studies are being performed in our laboratory to identify protein targets and susceptibility sites on proteins using mass spectrometry, research that may prove fruitful in identification of useful biomarkers of exposure and disease. Another goal is to develop highly sensitive laboratory assays that can be used to screen biological samples from workers and also for incorporation into hypothesis-based research on disease mechanisms.

In summary, we have successfully developed unique high affinity monoclonal antibodies that recognize dNCO–protein adducts resulting from conjugation with 2,4 or 2,6 TDI. The specificity of these antibodies was demonstrated by ELISA, Immunoblot and Dot blot analyses. Utility as potential tools for the identification of endogenous dNCO-modified proteins and the characterization of the disease relevant chemical linkages is also evident from results of our characterization and reactivity studies.

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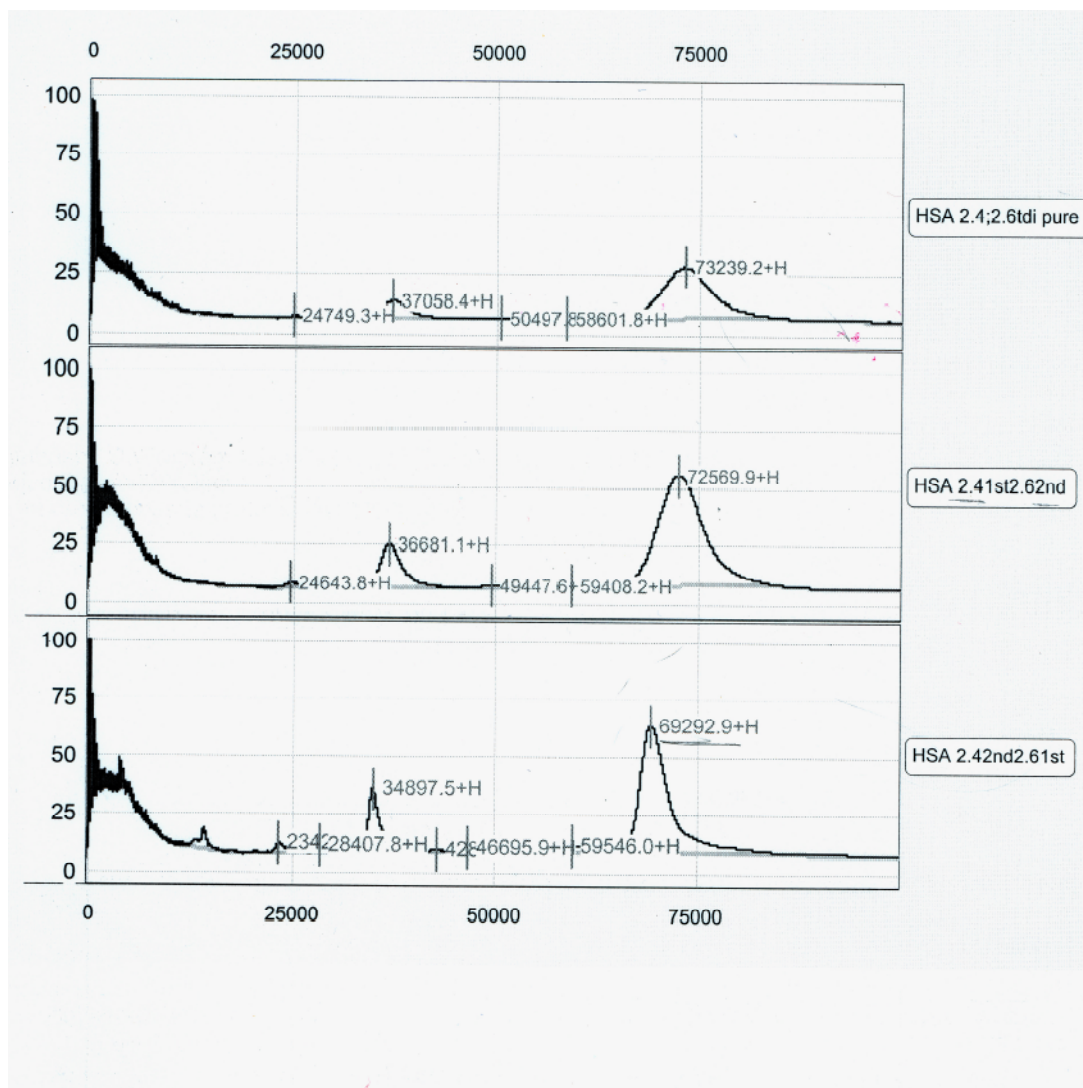
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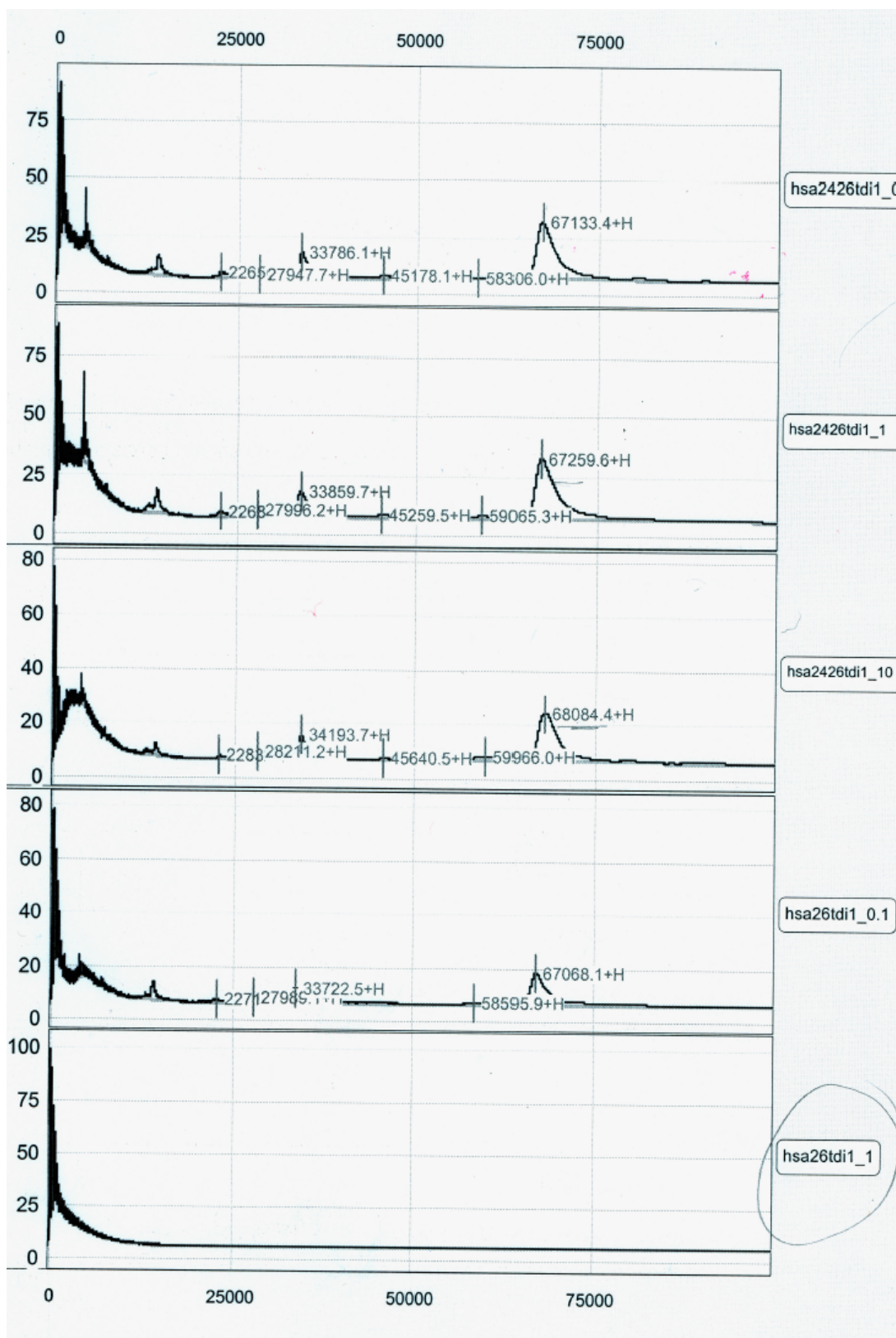
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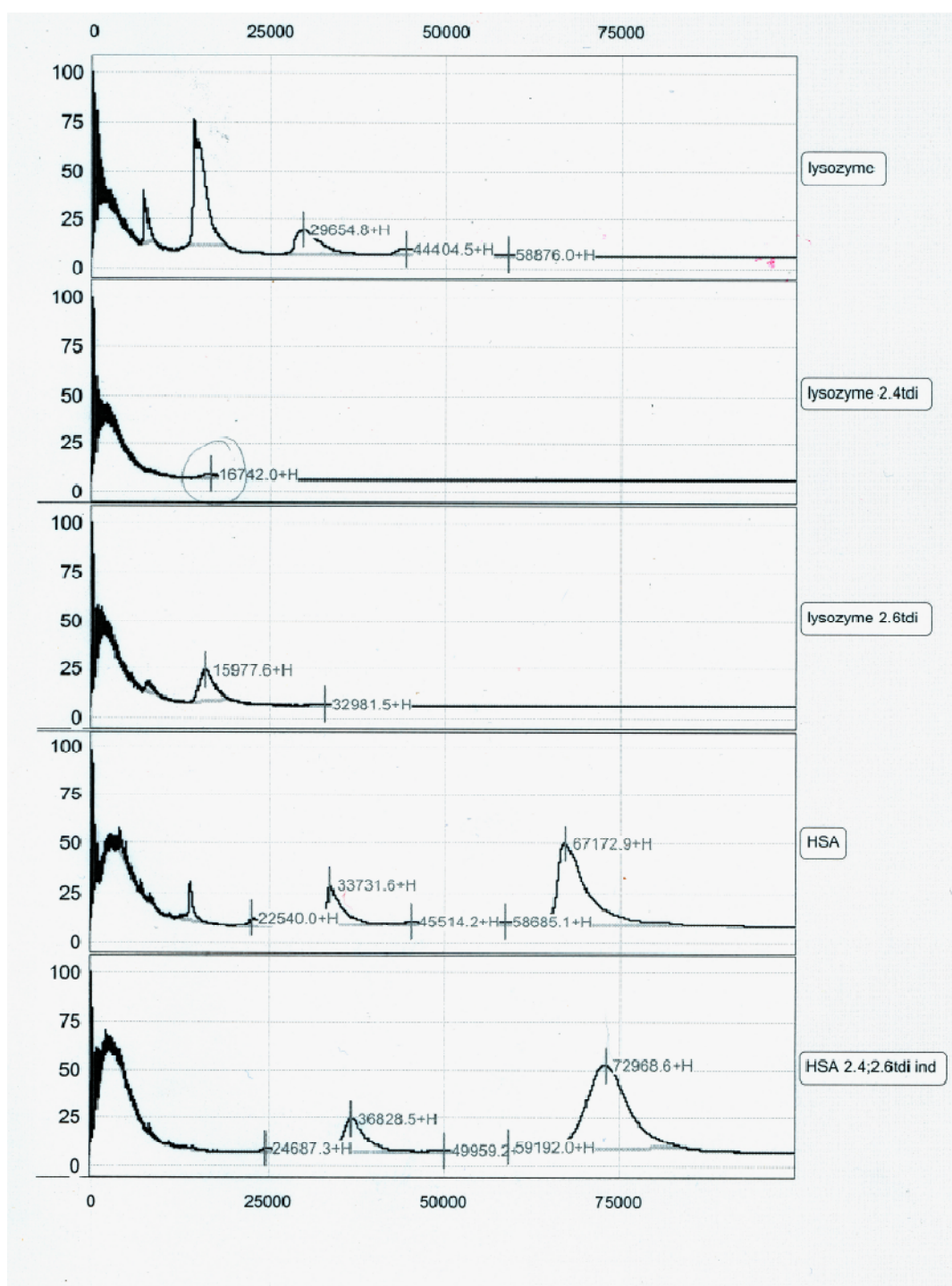
APPENDIX A

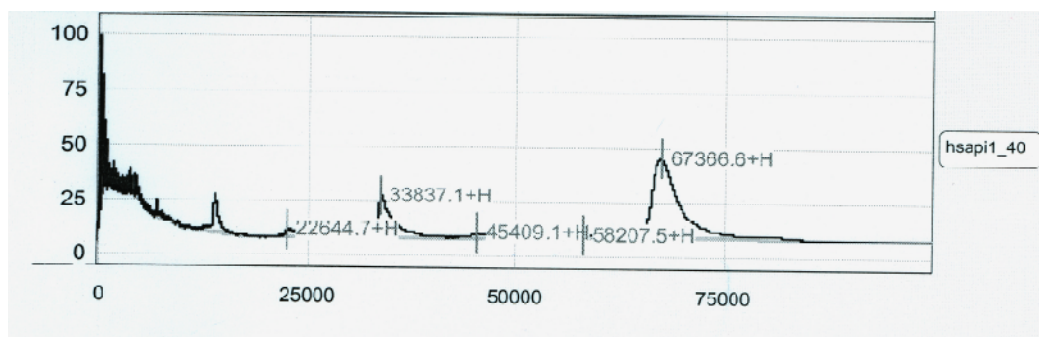
MALDI SPECTRA

Appendix A shows some of the spectra used to construct Table 3.1. The name of the conjugates and accompanying notes are on the right hand sides of the spectra.









APPENDIX B

TDI-VAPOR EXPOSED mABS CHARACTERIZATION

Table 1 showing 9 IgM mAbs reactivity with various isocyanate conjugates. The OD values are mean values of 4 well readings. 16F4, 29E6 and 56F9 (highlighted in gold) had reactivity to HSA that was less than 3 times the OD values of the blank indicating that their reactivity by ELISA to HSA (only) conjugate was minimal. Reactivity of 43B4 (highlighted in green) to 2,4 TDI-HSA was used a control for assay variability. There were no significant difference in the control values between plates and days of assay.

Table 1: ELISA reactivity of TDI-Vapor Mice

ELISA- Plate #:	1/2	3/4	5/6	7/8	9/10	11/12	13/14	15/16	17/18
mAb:	6C4	16F4	27G6	29E6	35D6	40C6	41G9	42C3	59F9
ANTIGEN	OD _{405 nm} VALUES								
HSA	0.386	0.059	0.591	0.065	4.458	0.242	0.559	0.412	0.156
2.4.-TDI-HSA, 1:40, no mAb (Control)	0.019	0.005	0.066	0.020	0.145	0.029	0.014	0.056	0.100
2.4.-TDI-HSA, 1:40	4.454	4.039	4.305	1.140	4.236	4.137	4.081	4.194	2.850
2.4.-TDI-HSA, 1:0.1	0.265	0.082	0.418	0.078	4.56	4.253	0.375	0.146	0.080
2.6.-TDI-HSA, 1:40	4.495	4.413	4.261	2.982	4.33	4.226	4.410	4.218	4.344
2.6.-TDI-HSA, 1:0.1	0.353	0.048	0.690	0.051	4.471	0.151	0.377	0.238	0.100
2.4, 2.6.-TDI-HSA, 1:40, industry mix	4.345	4.295	4.242	1.249	4.077	4.071	4.347	4.191	2.958
2.4., 2.6.-TDI- HSA, 1:40, pure mix	4.303	4.469	4.253	1.599	3.849	4.077	4.359	4.278	3.507
2.4., 2.6.-TDI- HSA, 1:40, pure mix	4.579	4.285	4.152	0.954	3.802	4.037	4.519	4.038	2.148
2.6., 2.4.-TDI	2.532	0.328	1.231	0.142	3.729	0.948	4.182	1.433	0.188

ELISA- Plate #:	1/2	3/4	5/6	7/8	9/10	11/12	13/14	15/16	17/18
mAb:	6C4	16F4	27G6	29E6	35D6	40C6	41G9	42C3	59F9
ANTIGEN	OD _{405 nm} VALUES								
1:40, mAb 43B4									

APPENDIX C

IgG mABS CHARACTERIZATION

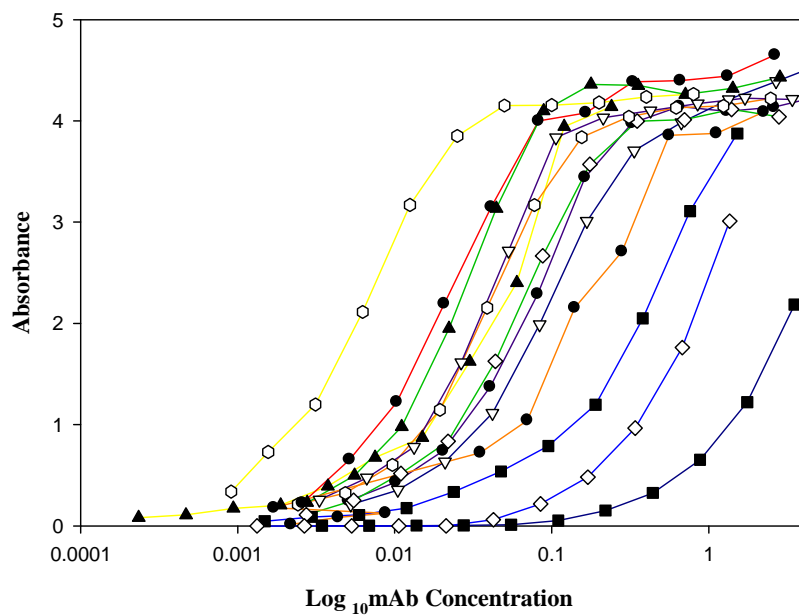


Figure 1: Titration curve for 2,6 TDI-KLH mAbs against 2,6 TDI-HSA to determine the concentration to be used for reactivity studies in Table 2 below. A similar curve was also constructed for Table 1.

Table 1 provides a grouping of 35 IgG mAbs produced from 2,4 TDI-KLH immunized mice into 11 groups based on similarity of reactivity pattern toward haptenated proteins. These groups were then cut down to 4 in the Table in Table 7.1 namely 2E5, 60G2, 62G5 and 79G7 based on overlapping and unique reactivities.

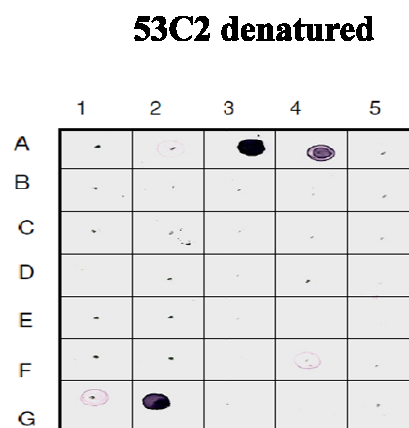
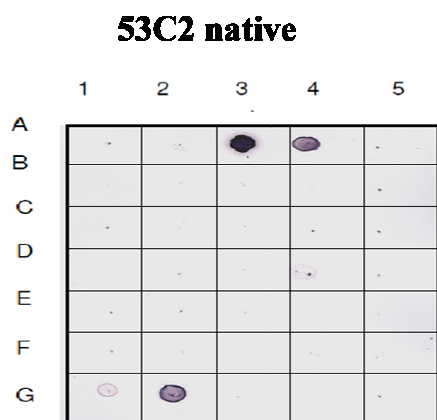
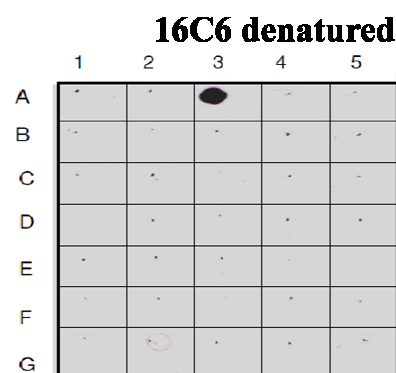
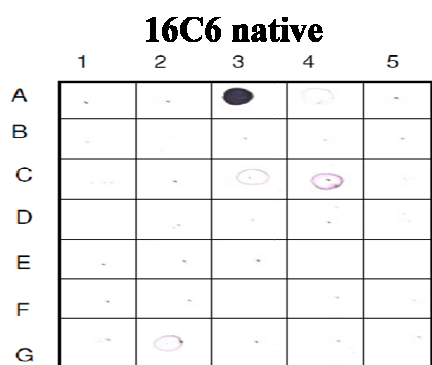
Table 2 provides the reactivity of 13 IgG mAbs produced from 2,6 TDI-KLH immunized mice. The mAbs have limited reactivity when compared with 2,4 TDI-KLH immunized mice (Table 1). Only 16C6, 32B6 and 59E5 are represented in Table 7.1. 43B3 was again used as the positive control for assay variability analysis. There were no significant inter-assay variabilities observed.

Table 1:ELISA data for 2,4 TDI-HSA screened mAbs

Chemical Name	Test Antigen ($\mu\text{g}/\text{mL}$)	Optical density [405 nm, 30 min, 10 $\mu\text{g}/\text{mL}$ mAb]											
		2E5 (λ)	43B4 (λ)	49B10 (λ)	57D5 (λ)	58E9 (λ)	66C2 (λ)	60D:0 (λ)	79G7 (λ)	62C5 (λ)	60G2 (λ)	62E4 (λ)	62E4 (λ)
2,4-toluene diisocyanate	2,4-TDI-HSA	2.70	2.93	0.76	3.25	2.40	1.26	4.01	3.20	2.88	3.57	0.04	0.04
	2,4-TDI-KLH	2.20	3.38	2.13	3.71	2.00	2.68	3.04	3.60	3.8	3.74	0.25	0.25
	2,4-TDI-MSA	2.57	2.54	1.27	3.56	2.72	2.01	4.04	3.62	3.21	3.61	0.09	0.09
	2,4-TDI-Albumin	1.08	2.36	0.17	1.03	2.93	0.38	3.83	1.51	0.53	3.11	0	0
	2,4-TDI-Isozyme	0	0	0	0	3	0	0	0	0	0	0	0
2,5-toluene diisocyanate	2,5-TDI-AS	0	0.65	0.07	3.11	0.14	0.09	0	2.6	3.07	3.66	0.36	0.36
	2,5-TDI-KLH	0	0.76	0.12	3.45	0.3	0.25	0.18	3.23	3.02	3.44	0.69	0.69
	2,5-TDI-MSA	0	0	0	0	3	0	0	0	0.24	0.1	0	0
	2,6 TDI keratin	0	0	0	0.27	3	0	0	0	1.72	0.12	0.05	0.05
	2,6-TDI-Isozyme	0	0	0	0	3	0	0	0	0	0		
4,4'-azobis(diphenyl diisocyanate)	MDI-HSA	0	0	0	0	3	0	0	0	0	0.66	0	0
hexamethylene diisocyanate	HDI-HSA	0	0	0	0	0.11	0	0	0	0	0.06	0	0
2,2'-bis[4-chlorophenyl]isocyanate	2,3-DMPI-HSA	0	0	0.07	0.64	3	1.44	0	3.8	3.84	0	0	0
2,5-bis[4-chlorophenyl]isocyanate	2,5-DMPI-HSA	0	0	0	0	0.41	0.06	0	1.54	2.19	0	0	0
3,4-bis[4-chlorophenyl]isocyanate	3,4-DMPI-HSA	0	0	0	0	0.27	0.08	0	0.61	0.77	0	0	0
4-toluene isocyanate	PTI-HSA	0	0	0	0	3	0	0.05	0	0	0.05	0	0
2-toluene isocyanate	OTI-HSA	0	0	0	0	3	0	0	1.02	3.2	0	0	0
phenyl isocyanate	PI-HSA	0	0	0	0	3	0	0	0.06	0	0	0	0
2,4-toluene diisocyanate	2,4'-TTC-HSA	0	0	0	0	3	0	0.62	0	0	0	0	0
2,6-toluene diisocyanate	2,6'-TTC-HSA	0	0	0	0	3	0	0	0	0	0	0	0

Protein	Optical density [405 nm, 30 min]												
	16C 6	31F 2	32B 6	53C 2	53C 6	54F 8	57G 8	59E 5	60C 5	60C1 1	68D 3	68D 5	68E 4
2,4 TITC-HSA	0.07	0.0 3	0.0 1	0.05	0.03	0.0 5	0.02	0.0 0	0.03	0.03	0.01	0.01	0.0 8
2,6 TITC-HSA	0.06	0.0 3	0.0 5	0.02	0.04	0.0 4	0.02	0.0 1	0.08	0.01	0.02	0.02	0.0 2
MSA	0.09	0.0 3	0.1 5	0.13	0.12	0.0 6	0.10	0.1 5	0.11	0.08	0.16	0.15	0.1 4
collagen	0.02	0.0 4	0.0 4	0.02	0.02	0.0 5	0.02	0.0 3	0.02	0.01	0.02	0.01	0.0 1
keratin	0.03	0.0 4	0.0 3	0.02	0.02	0.1 0	0.02	0.0 1	0.04	0.01	0.10	0.01	0.0 2
lysozyme	0.02	0.0 4	0.0 4	0.03	0.02	0.0 3	0.04	0.0 5	0.01	0.01	0.01	0.02	0.0 2
KLH	0.04	0.0 3	0.0 3	0.00	0.02	0.0 3	0.02	0.0 4	0.04	0.00	0.00	0.01	0.0 4
2.4. TDI-HSA, 1:40, mAb 43B4	3.77	3.5 6	3.7 8	3.28	3.98	3.9 9	3.66	4.0 3	3.55	3.46	4.13	3.86	3.7 6

Dot ELISA blots for mAbs 16C6, 53C2, 59E5, 68D5, 2E5, 43B4, 60G2 and 62G5.



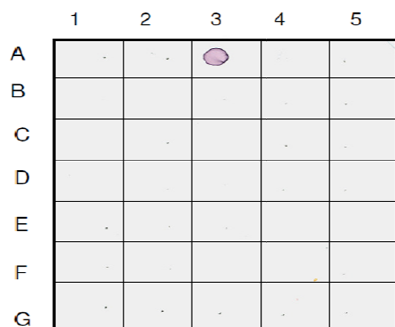
KEY:

Lane A1: HSA
Lane A4: HSA-2,4;2,6 TDI
Lane B2: HSA-Gorrilla glue
Lane B5: HSA-OTI
Lane C3: HSA-3,5 DMPI
Lane D1: AHSA-2,4 TDI
Lane D4: KLH-2,4 TDI
Lane E2: Lysozyme-2,4 TDI
Lane E5: Collagen-2,4 TDI
Lane F3: Keratin-2,4 TDI
Lane G1: MSA-2,4 TDI
Lane G4: CH3NH -2,4 TDI

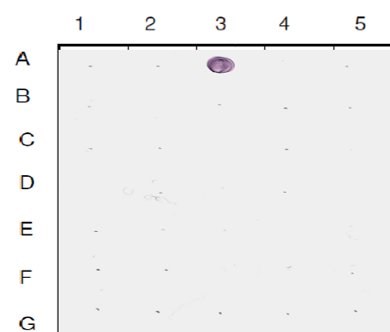
Lane A2: HSA-2,4 TDI
Lane A5: HSA-HDI
Lane B3: HSA-2,4 TITC
Lane C1: HSA-PTI
Lane C4: HSA-2,5 DMPI
Lane D2: AHSA-2,6 TDI
Lane D5: KLH-2,6 TDI
Lane E3: Lysozyme-2,6 TDI
Lane F1: Collagen 2,6 TD
Lane F4: Keratin-2,6 TDI
Lane G2: MSA-2,6 TDI
Lane G5: CH3NH-2,6 TDI

Lane A3: HSA-2,6 TDI
Lane B1: HSA-MDI
Lane B4: HSA-2,6 TITC
Lane C2: HSA-PI
Lane C5: AHSA
Lane D3: KLH
Lane E1: Lysozyme
Lane E4: Collagen
Lane F2: Keratin
Lane F5: MSA
Lane G3: CH3NH2

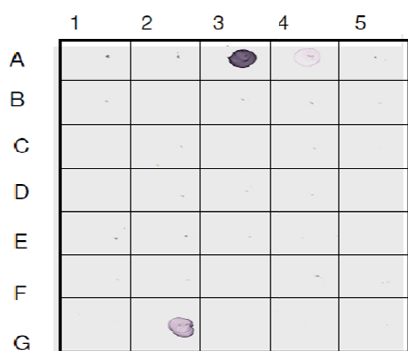
59E5 native



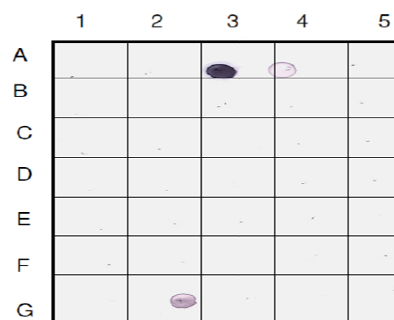
59E5 denatured



68D5 native



68D5 denatured



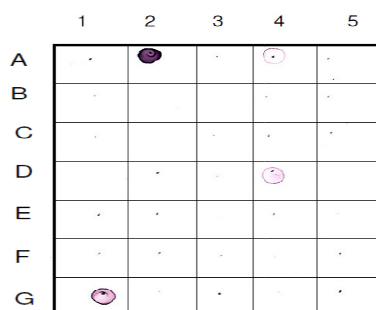
Key:

Lane A1: HSA
Lane A4: HSA-2,4;2,6 TDI
Lane B2: HSA-Gorilla glue
Lane B5: HSA-OTI
Lane C3: HSA-3,5 DMPI
Lane D1: AHSA-2,4 TDI
Lane D4: KLH-2,4 TDI
Lane E2: Lysozyme-2,4 TDI
Lane E5: Collagen-2,4 TDI
Lane F3: Keratin-2,4 TDI
Lane G1: MSA-2,4 TDI
Lane G4: CH3NH⁻-2,4 TDI

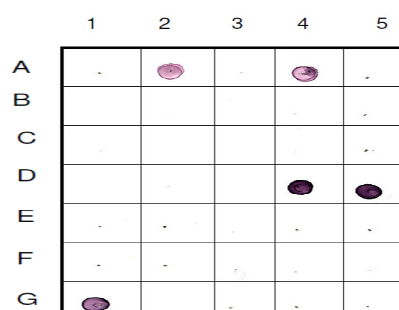
Lane A2: HSA-2,4 TDI
Lane A5: HSA-HDI
Lane B3: HSA-2,4 TITC
Lane C1: HSA-PTI
Lane C4: HSA-2,5 DMPI
Lane D2: AHSA-2,6 TDI
Lane D5: KLH-2,6 TDI
Lane E3: Lysozyme-2,6 TDI
Lane F1: Collagen 2,6 TD
Lane F4: Keratin-2,6 TDI
Lane G2: MSA-2,6 TDI
Lane G5: CH3NH⁻-2,6 TDI

Lane A3: HSA-2,6 TDI
Lane B1: HSA-MDI
Lane B4: HSA-2,6 TITC
Lane C2: HSA-PI
Lane C5: AHSA
Lane D3: KLH
Lane E1: Lysozyme
Lane E4: Collagen
Lane F2: Keratin
Lane F5: MSA
Lane G3: CH3NH₂

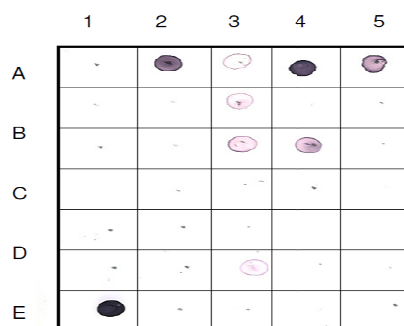
2E5native



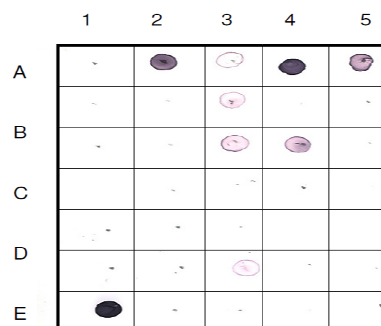
2E5 denatured



43B4 native



43B4 denatured



KEY: **Lane A1:** HSA
Lane A4: HSA-2,4;2,6 TDI
Lane B2: HSA-Gorilla glue
Lane B5: HSA-OTI
Lane C3: HSA-3,5 DMPI
Lane D1: AHSA-2,4 TDI
Lane D4: KLH-2,4 TDI
Lane E2: Lysozyme-2,4 TDI
Lane E5: Collagen-2,4 TDI
Lane F3: Keratin-2,4 TDI
Lane G1: MSA-2,4 TDI
Lane G4: CH3NH -2,4 TDI

Lane A2: HSA-2,4 TDI
Lane A5: HSA-HDI
Lane B3: HSA-2,4 TITC
Lane C1: HSA-PTI
Lane C4: HSA-2,5 DMPI
Lane D2: AHSA-2,6 TDI
Lane D5: KLH-2,6 TDI
Lane E3: Lysozyme-2,6 TDI
Lane F1: Collagen 2,6 TD
Lane F4: Keratin-2,6 TDI
Lane G2: MSA-2,6 TDI
Lane G5: CH3NH-2,6 TDI

Lane A3: HSA-2,6 TDI
Lane B1: HSA-MDI
Lane B4: HSA-2,6 TITC
Lane C2: HSA-PI
Lane C5: AHSA
Lane D3: KLH
Lane E1: Lysozyme
Lane E4 : Collagen
Lane F2: Keratin
Lane F5: MSA
Lane G3: CH3NH2