CELLULAR MODELS FOR ENVIRONMENTAL TOXICANT BIOMARKER DISCOVERY

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ABSTRACT

A research and development priority in the area of occupational environmental health surveillance (OEHS) is the development of biomarkers of exposure, effect, and susceptibility (1). As exposure monitoring using environmental sampling equipment can be impractical and doesn't account for differences in individual responses, new methodologies must be sought. Biomarkers offer the opportunity to quantify toxic exposures and effects in individuals before, during, and after deployments. Therefore, concerted efforts in this area of research are underway at the U.S. Army Center for Environmental Health Research (USACEHR). This report demonstrates the feasibility of using *in vitro* cellular models as biomarker discovery tools. The combination of these models with state of the art proteomic screening technologies and an enzyme-linked immunosorbent assay (ELISA), provides insight into the effects of trinitrotoluene (TNT), dinitrobenzene (DNB), and the carbamate aldicarb. The results of these toxicant exposures are discussed.

1. INTRODUCTION

The operational environments that our Soldiers, Sailors, Airmen and Marines often encounter can expose them to an array of Toxic Industrial Chemicals (TICs), Toxic Industrial Materials (TIMs), and Military Relevant Chemicals (MRCs). Many of these toxicants are very hazardous and could seriously affect the health and mission effectiveness of our troops. Therefore, it is essential to identify the occurrence of environmental hazard exposures to our troops, and to determine whether or not these exposures may result in subsequent adverse health effects. Biomarkers, which are physiological or biochemical indicators of exposure, effect, or susceptibility, have the potential to provide this type of information.

USACEHR, in collaboration with the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), is currently developing *in vitro* cellular models for biomarker discovery. USACEHR and USAMRIID are leveraging their individual strengths by applying both proteomic and functional genomic screening methods, as well as interrogating the proinflammatory cytokine and chemokine responses of human peripheral blood mononuclear cells (PBMC) to environmental toxicants (Fig. 1). An immortalized human hepatocellular (HepG2) cell model is also being developed to evaluate the effects of these environmental toxicant exposures.

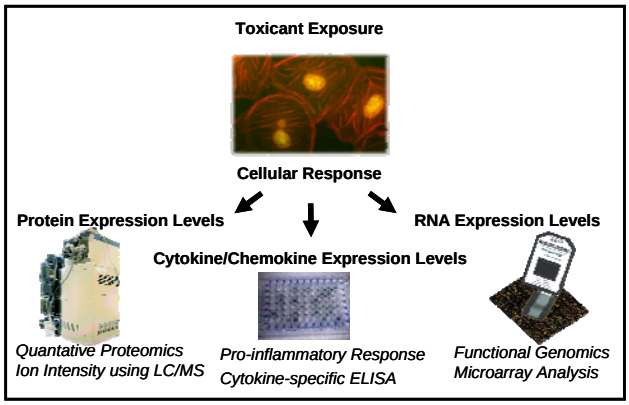

Fig. 1 – Biomarker discovery *in vitro* model development.

Initial work explores the effects of MRCs such as TNT, DNB, and other environmental toxicants such as aldicarb. This paper discusses model development, the latest advances in screening methodologies, and the data analysis tools used in our biomarker discovery efforts. Additionally, potential future applications of novel protein and/or nucleic acid biomarkers discovered during this effort will be presented.

2. METHODS

The methods described here advanced the *in vitro* biomarker discovery project at USACEHR. One of the key requirements for a clinically useful biomarker in humans is that it should be easily accessible, such as in serum, plasma, urine or saliva. For this reason, human PBMC are an attractive choice for exposure response investigations. Additionally, in an effort to identify markers indicative of general toxicity, HepG2 cells were

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exposed for short periods to varying concentrations of aldicarb to investigate rapid cellular responses for potential future monitoring. The proposed *in vitro* strategy takes a three tiered approach to characterizing new biomarkers, discovering novel protein and RNA biomarkers and assessing cytokine/chemokine expression levels. Only two of these, the protein and cytokine/chemokine expression levels, will be discussed here.

2.1 Peripheral Blood Mononuclear Cell Exposures

Human PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from randomly selected, healthy donors. PBMC were cultured in 6-well plates at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) at a concentration of 10^6 cells/mL. Cells were either quiescent or stimulated with Staphylococcal Enterotoxin B (SEB) (300 ng/mL) immediately after addition of either TNT or DNB. The concentrations of both TNT and DNB were set at 5, 50, and 500 times the Military Exposure Guideline (MEG) concentrations. This equates to 0.3, 3.0, and 30 mg/L for TNT, and 0.15, 1.5, and 15 mg/L for DNB. Controls included both quiescent and SEB stimulated PBMC in FCS supplemented DMEM. Following a 22-24 hr exposure, the medium was removed and centrifuged to remove suspended cells. The supernatants were analyzed to determine concentrations of TNF-α, IL-6, IL-2, IFN-γ, MCP-1, MIP-1α, and MIP-1ß by enzyme-linked immunosorbent assay (ELISA) with cytokine- or chemokine-specific antibodies, as previously described (2, 3). Human recombinant cytokines and chemokines (20 to 1,000 pg/ml) were used as calibration standards for each plate. The detection limit for these assays was 20 pg/mL.

After removal of the supernatants, the attached cells were harvested, pooled with the suspended cell pellet obtained after centrifugation of the supernatants, washed twice with Phosphate Buffered Saline (PBS), and lysed using the QIAshredder and Qiagen RNeasy Kit (Qiagen Inc., Valencia, CA). The RNA and proteins were isolated from the lysate according to the manufacturer's recommendations. After tryptic digestion, the peptides were analyzed by liquid chromatography/ mass spectrometry (LC/MS^E) on a Waters quadrupole time-of-flight (Q-TOF) Premier mass spectrometer coupled to a NanoAcquity ultra performance liquid chromatography (UPLC) system (Waters Corp., Milford, MA).

2.2 HepG2 Hepatoma Cell Exposure

HepG2 cells, passage 76, were used in these experiments and were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained at 37°C in DMEM complete culture media in T75 culture flasks. Complete culture media contains DMEM (500 mL) with 4.5 g/L glucose and L-glutamine supplemented with 10% Fetal Bovine Serum (FBS), 20 mM HEPES Buffer, and 100 units Penicillin and 100 µg Streptomycin.

HepG2 cells were seeded in 6-well plates, fresh DMEM complete culture media was added, and the plates were incubated for 48-72 hr until a confluent monolayer was obtained. Prior to toxicant exposure, the medium was removed by aspiration and replaced with serum-free, antibiotic-free DMEM with 4.5 g/L glucose and L-glutamine. The cells were incubated for at least 1 hr at 37°C to ensure cell stability prior to toxicant exposure. The culture media was aspirated and replaced with serum-free, antibiotic-free DMEM with 4.5 g/L glucose and L-glutamine or DMEM containing 1, 10 and 100 mg/L of aldicarb. After exposing the cells for 1 hr, they were washed twice with PBS, harvested and lysed, using the RNeasy Kit. The RNA and proteins were isolated from the lysate. After tryptic digestion, the peptides were analyzed by LC/MS^E on a Waters Q-TOF Premier mass spectrometer coupled to a NanoAcquity UPLC system.

2.3 Mass Spectrometry

Following tryptic digestion, the peptides were separated with reverse phase chromatography using a Waters NanoAcquity UPLC coupled to a Waters Q-TOF Premier Mass Spectrometer.

Table 1. Time Table Program for the Analytical Separation of Aldicarb Peptides

NanoAcquity Pump Program			
Time (minutes)	Solvent		Flow Rate µL/min
	0.1% Formic acid in Water	0.1% Formic acid in Acetonitrile	
	Percentage of Composition		
0	97	3	0.25
1	97	3	0.25
101	70	30	0.25
105	50	50	0.25
106	80	15	0.25
111	80	15	0.25
112	97	3	0.25
Stop time 140 minutes	97	3	0.25

The aldicarb samples were analyzed prior to an upgrade to the UPLC system and had different analytical conditions. The peptides resulting from the aldicarb exposure were trapped using a Waters Symmetry C18 180 µm X 20 mm, 5 µm particle size column using a 5 µL/ min flow rate for 4 minutes using 0.1% formic acid. The analytical separation for aldicarb was performed using a Waters NanoAcquity UPLC column BEH C18 75 µm X 100 mm, 1.7 µm particle size. The gradient table for this separation is shown in Table 1. The injection volume was 10 µL and the analytical column was maintained at 35°C.

Table 2. Time Table Program for the Analytical Separation of TNT and DNB Peptides

NanoAcquity Pump Program			
Time (minutes)	Solvent		Flow Rate µL/min
	0.1% Formic acid in Water	0.1% Formic acid in Acetonitrile	
	Percentage of Composition		
0	99	1	1.2
1	99	1	1.2
101	70	30	1.2
105	50	50	1.2
106	85	15	1.2
111	85	15	1.2
112	99	1	1.2
Stop time 140 minutes	99	1	1.2

The peptides for the TNT and DNB exposures were trapped using a Waters Symmetry C18 180 µm X 20 mm, 5 µm particle size column using a 5 µL/min flow rate for 6 minutes using 0.1% formic acid. The analytical separations for DNB and TNT were performed using a Waters NanoAcquity UPLC column BEH 130 C18 100 µm X 100 mm, 1.7 µm particle size. The gradient table for this separation is shown in Table 2. The injection volume was 9.9 µL and the analytical column was maintained at 35°C.

Peptides were detected using a Waters Q-TOF Premier. The Q-TOF premier is a quadrupole, orthogonal acceleration time-of-flight tandem mass spectrometer. The peptides were ionized using electrospray ionization in positive ion mode. Data was collected over the 50-1900 m/z range. Data for the high and low energy scans were collected for 0.8 seconds. An external lock mass was used for calibrating mass accuracy. A lockmass reading was taken every 30 seconds using a 1 second scan.

2.4 Database Searches

Raw data was extracted and database searches were performed using Protein Lynx Global Server 2.2 (PLGS2.2). The database used was created from the entire National Center for Biotechnology Information (NCBI) human Reference Sequence (RefSeq) protein database (www.ncbi.nlm.nih.gov) supplemented with possible contaminants, including human keratins, bovine serum albumin (BSA), and trypsin. Random sequences greater than or equal in number to the RefSeq data were added to the database as a control for false positives.

3. RESULTS

3.1 HepG2 Hepatoma Protein Analysis

Analysis of the peptide identifications obtained from the mass spectrometry data from three separate replicates resulted in the identification of 21 proteins with changes in abundance when compared to the control as a result of aldicarb exposure. Of these 21 proteins, five had a greater than ~1.5 fold difference following the one hour exposure. Table 3 shows that three proteins decreased in abundance and one increased at 100 mg/L aldicarb. One protein decreased in abundance at 1 mg/L aldicarb.

Table 3. Aldicarb Induced HEPG2 Protein Changes

Accession Number	Description	Ratio - Control: Aldicarb	Aldicarb Concentration
4505763	phosphoglycerate kinase 1	1.9155	100 mg/L
29788785	tubulin beta polypeptide	1.5373	100 mg/L
57013276	tubulin alpha ubiquitous	1.6653	100 mg/L
20070125	prolyl 4 hydroxylase beta subunit	0.6771	100 mg/L
4505621	prostatic binding protein	1.6487	1 mg/L

3.2 PBMC proinflammatory cytokine and chemokine response

The PBMC exposures examine the possible immunosuppressive effects of toxicants by stimulating PBMC simultaneously with a superantigen, SEB. SEB stimulates PBMC by binding to MHC class II and T-cell-receptors resulting in production of inflammatory cytokines. The results of cytokine and chemokine measurements in the supernatant of SEB plus toxicant exposed PBMC are shown in figures two and three. For both the TNT (Fig. 2) and the DNB (Fig. 3) exposures, we assayed the concentrations of the cytokines TNFα, IL-6, IFNγ, IL-2, MCP-1, MIP-1α and MIP-1β by ELISA.

Interestingly, neither of the toxicants, TNT nor DNB, by themselves stimulates PBMC to produce proinflammatory cytokines and chemokines. For this reason, PBMC were stimulated with SEB, which does induce a response, in order to investigate the effects of toxicant exposure. The results of TNT and DNB exposure on SEB-stimulated PBMC cytokine and

chemokine responses are shown in Fig. 2 and Fig. 3 respectively. Each assayed cytokine appears to decrease in concentration with increasing toxicant concentration. This is most apparent for the chemokines. This could suggest that both TNT and DNB inhibit the PBMC proinflammatory response.

TNT PBMC EXPOSURE

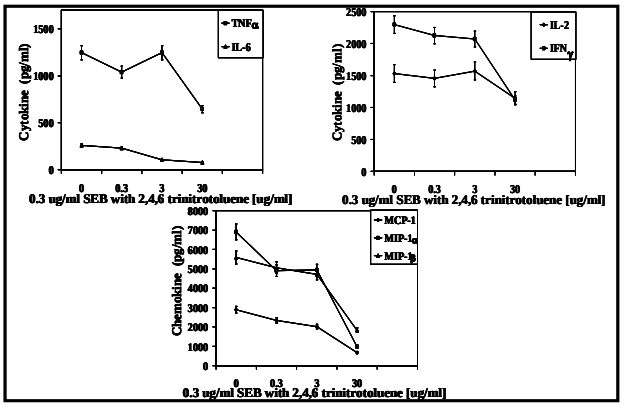

Fig. 2 – SEB-stimulated PBMC proinflammatory response following exposure to TNT. PBMC from one donor stimulated with 0.3 μg/mL SEB were exposed to 0.0, 0.3, 3.0, and 30 mg/L TNT for 22-24 hr.

DNB PBMC EXPOSURE

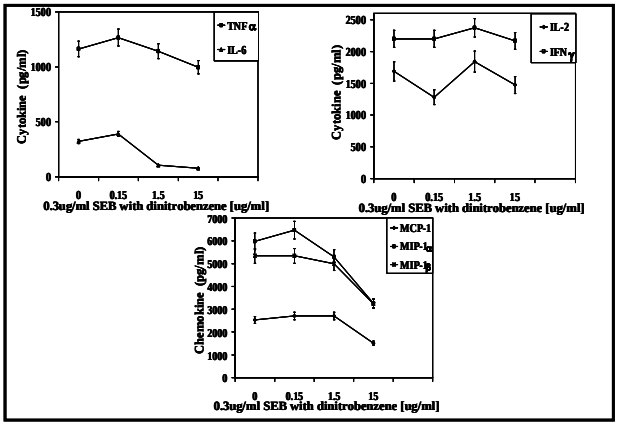

Fig. 3 – SEB-stimulated PBMC proinflammatory response following exposure to DNB. PBMC from one donor stimulated with 0.3 μg/mL SEB were exposed to 0.0, 0.15, 1.5, and 15 mg/L DNB for 22-24 hr.

CONCLUSIONS

This report demonstrates that the i*n vitro* models described here and the experimental approaches used to implement these models have the potential to aid in the identification of biomarkers indicative of environmental toxicant exposure and the possible effects of these exposures on the warfighter. In addition, we show that quantitative proteomics using ion intensity is a feasible method for identification and quantification of protein differences in exposed vs. unexposed cells. Lastly, our preliminary results suggest that both TNT and DNB may suppress SEB-stimulated cytokines and chemokines, and that these toxicants may act as immunosuppressants.

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Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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