Technical Evaluation Report

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1.0 INTRODUCTION.

The threat of chemical or biological weapons use as a means of countering conventional military superiority has provided a renewed interest in chemical and biological defense. As a result of the renewed interest and of recent concerns regarding possible terrorist use of these weapons of mass destruction (WMD), civilian health authorities in many nations have turned to their military CB experts for potential solutions to this new menace. Of course, requirements for protecting military forces continue. In response, there have been many changes in this area in the years since the last Human Factors and Medicine (HFM) panel meeting addressing the medical and operational toxicological aspects of chemical warfare agents. That meeting occurred outside Lisbon, Portugal in 2001. These changes since 2001 include epidemiological or clinical studies of exposed or potentially exposed populations, new treatment concepts and products, improved organization of the national response apparatus of many member nations, and improved diagnostic tests that enable rapid diagnosis and treatment.

To address the developments sparked by event since 2001, NATO representatives from 29 nations met in Edinbourgh, Scotland on 8-10 October 2007 to attend a symposium on Defense Against the Effects of Chemical Hazards: Toxicology, Diagnosis, and Medical Countermeasures arranged by NATO/RTO/HFM. One of the core mission areas addressed by the Human Factors and Medicine Panel is protection in adverse environments (HP) – i.e., human-centered research for optimizing physiological tolerance, protection and survivability in adverse mission environments, in the case of the Edinbourgh meeting -- a chemically contaminated environment. The Symposium reviewed state-of-the-art prophylactic, diagnostic, and therapeutic countermeasures, the increasing threat of Toxic Industrial Chemicals (TICs) and Toxic Industrial Materials (TIMs), and discuss future challenges in medical chemical defense. All of these topics were reviewed within the framework of NATO and National policies, programs and capabilities to address the requirement for potential operations in a chemical environment. To the greatest extent possible, each session was co-moderated by a member of the HFM Panel and a member of the Joint Medical Committee.

For purposes of summarizing the subject matter the 31 Papers, 11 Posters, and 2 Keynote addresses can be grouped into seven subject areas: NATO/National Policies, Programs and Response to WMD Use; mustard and nerve agent toxicology; diagnostics of OP exposure; toxins; OP medical treatments; operational toxicology; and future of medical countermeasures.

2.0 NATO / NATIONAL POLICIES, PROGRAMS AND RESPONSE TO WMD USE

- "NATO WMD Initiative: The Way Forward," Mr. E.C. Whiteside, Director, NATO Weapons of Mass Destruction Centre, NATO HQ, BE

- "Medical Countermeasures to Weapons of Mass Destruction: The NATO Advantage," Murray G. Hamilton, Ph.D., CA

These two Keynote Addresses set the stage, and the tone, for the Symposium.

2.1. "NATO WMD Initiative: The Way Forward," Mr. E.C. Whiteside, Director, NATO Weapons of Mass Destruction Centre, NATO HQ, BE.

Mr. Ted Whiteside's overview of the NATO WMD Initiative set a policy framework for the subject of WMD science, technology and operations. In his talk he described NATO's tools to counter the proliferation of WMD—viz., the Chemical Weapons Convention (CWC), the BTWC, the Nuclear Non-Proliferation Pact (NNP), and NATO's new deployable CBRN defense battalions. He also suggested that there exists the possibility of a "virtual" pharmaceutical stockpile and that the relationships among NATO HFM, OPCW, and other initiatives should enable NATO commanders to "Reach Back" to national experts for technical advice. These NATO initiatives have national program underpinnings in defense preparedness and civil protection. The latter competencies are based in national research programs and their current medical countermeasures advanced development efforts.

2.2. "Medical Countermeasures to Weapons of Mass Destruction: The NATO Advantage," Murray G. Hamilton, Ph.D., CA.

Murray Hamilton (CAN) presented a broad-ranging overview of past, current, and potential future developments within CB defense. In the area of nerve agents he described pre-treatment approaches such as pyridostigmine, physostigmine/Hyoscine combinations, BuChAse (scavenging). With respect to immediate therapy for nerve agents he discussed oximes, neuroprotectants, medical devices, and autoinjector technologies. He pointed out the evolutionary nature of this review, one example of which is that the need for neuroprotectants becomes more apparent when one has developed improved life-saving therapies. He discussed the promise of new approaches such as combinatorial chemistry, peptide libraries, and peptide mimetics. Dr Hamilton pointed out two of the significant challenges to this community – it is small and its products are orphan products. Thus, its research is orphan research. Because of these challenges collaborative efforts are essential.

2.3. Summary.

In this summary of the Symposium we also endeavor to discuss future challenges in medical chemical defense, the future of medical countermeasures against CWA, and how the development of these future medical countermeasures would be enhanced by the application of state-of –the-art technologies.

3.0. MUSTARD AND NERVE AGENT TOXICOLOGY

- "Temporal Expression of Neuro-inflammatory Markers and Brain Injury Following Acute Soman Poisoning, R.K. Kan, et al, USA"
- "Soman-Induced Neuro-Inflammatory Reaction in Mouse Brain: Some Effects of a Combination of Atropine and Ketamine," F. Dhote, et al, FRA
- "An Animal Model to Study Health Effects during Long-Term Low –Dose Exposure to Toxic Agents," Cassel et al, SWE
- "Gene Expression Responses to Sulphur Mustard," Ford et al, CAN
- "Gene-Expression Profiles of Vesicant-Induced Skin Injury," Gerecke et al, USA

- "Inhalation Toxicology of Sulphur Mustard: Dose-Ranging Study in a Porcine Model." Jugg et al, GBR
- "The Effect of Anaesthetic and Oxygen Tension on the Toxicity of OP Nerve Agents," Sawyer et al, CAN (Poster)
- "Genomic Analysis of Pulmonary Toxicants to Identify Mechanisms of Tooxicity and Strategies for the Development of Medical Counermeasures," Dillman et al, USA (Poster)
- "Location of the Sulphur Mustard Skin Reservoir in Vitro," Dalton et al, GBR (poster)
- "The Effect of Percutaneous Administration of VX in the Freely-Moving Guinea Pig," Mumford et al, GBR (poster)

3.1. **"Temporal Expression of Neuro-inflammatory Markers and Brain Injury Following Acute Soman Poisoning, R.K. Kan, et al, USA"**

Kan et al (USA) opened the session by examining the temporal expression of inflammatory mediators (chemokines and cytokines) in the piriform cortex, correlated these findings with brain pathology for the purposes of determining the therapeutic window for attenuation of nerve agent-induced brain injury. They found a correlation between markers of inflammation and the therapeutic window for anti-inflammatory treatment using decrease in MAP-2 fluorescence probe uptake as a marker of neurodegeneration. No MAP-2 loss was observed for at least 6 hours, suggesting this time period as a window for therapeutic intervention. See Figure 1.
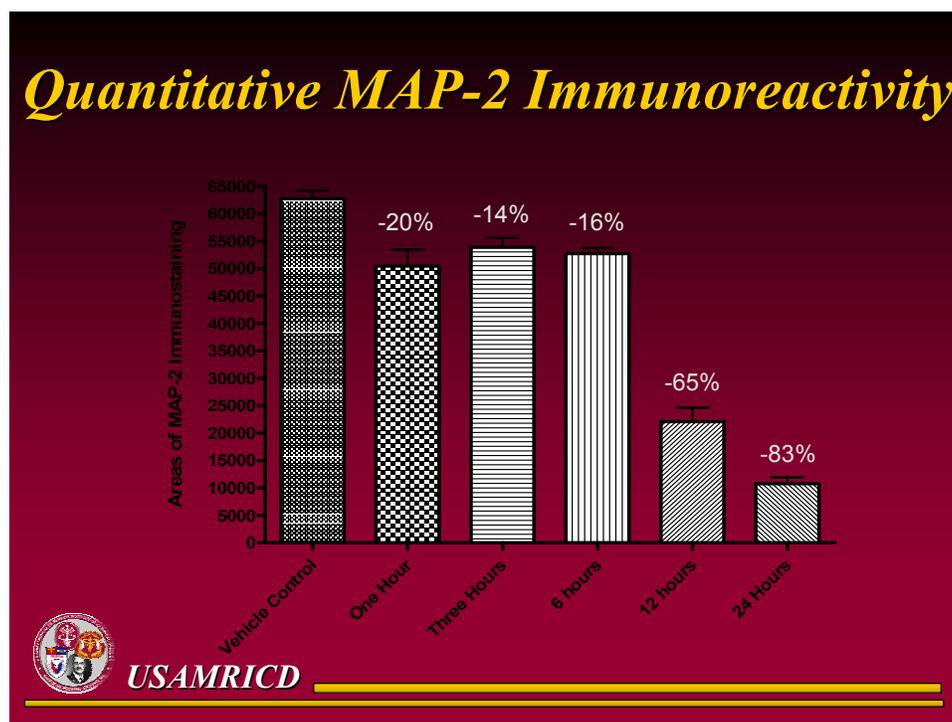

Figure 1. Time course of MAP-2 immunoreactivity where decrease in MAP-2 uptake represents neurodegeneration, from Kan et al, USA.

3.2. "Soman-Induced Neuro-Inflammatory Reaction in Mouse Brain: Some Effects of a Combination of Atropine and Ketamine," F. Dhote, et al, FRA

Conversely, Dhote et al (FRA) undertook a quantitiatve RT-PCR analysis of the brain gene expression responses to soman (viz., IL-1B, TNF-a, IL-6, and SOC3) for up to seven days after soman poisoning in mice. Changes in mRNA levels were quantified in hippocampus, cortex and cerebellum. The authors also assessed the effects of a ketamine/atropine combination at 48 hrs. Activation of gene expression was detected as early as one hr post-intoxication with peak response occurring at between 24 and 48 hours and persisting for 7 days. The ketamine/atropine combination was also shown to attenuate the soman-induced increase in mRNA.

3.3. "An Animal Model to Study Health Effects during Long-Term Low –Dose Exposure to Toxic Agents," Cassel et al, SWE

Cassell et al (SWE) attempted to characterize long-term low dose exposure to toxic agents, using VX as their probe. They presented a model of freely-moving, awake male Wistar rats, given a sustained subcutaneous release of VX accompanied by a weekly blood draw for analysis of exposure biomarkers. The author made an important distinction between biomarkers of exposure and biomarkers of effect and gave examples of each (see Figure 2).
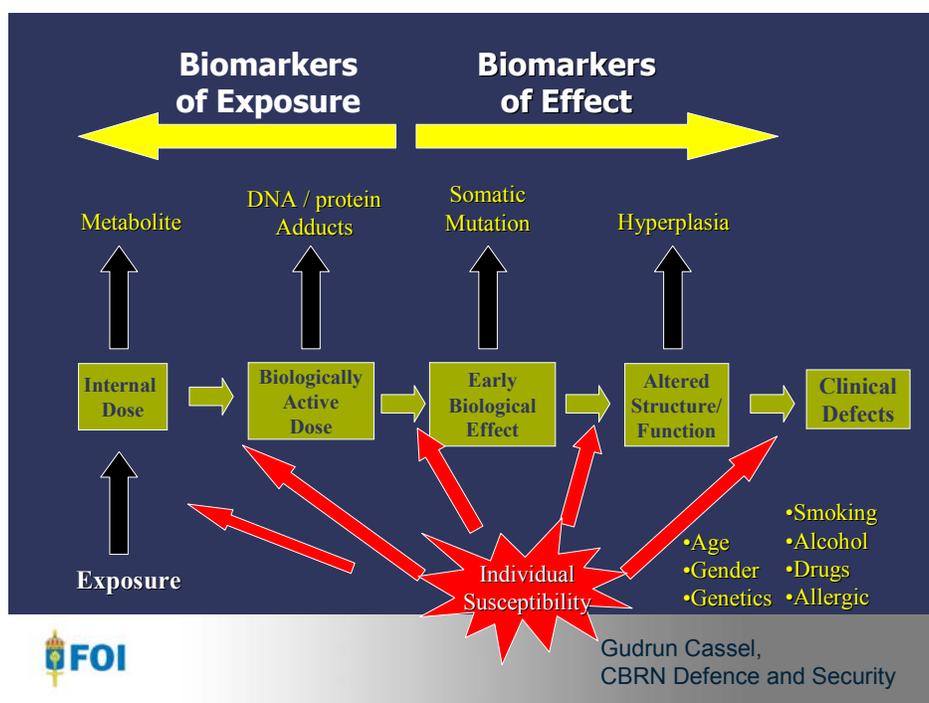

Figure 2. The notion of biomarkers and their applications, from Cassell et al SWE.

3.4. "Gene Expression Responses to Sulphur Mustard," Ford et al, CAN

On the sulfur mustard (SM) side, Ford et al (CAN) conducted a 21K microassay gene expression analysis of primary cell culture of normal human epidermal keratinocytes (NHEKs). Ford et al reported that at

concentrations exceeding 200 μM HD, total RNA yield and quality from harvested cells is consistently reduced. This suggests that global transcription is substantially affected, and that some published data are actually measures of relative RNA degradation rather than modulated gene expression. At lower doses of HD, ontology and pathway analysis of early gene expression responses led them to investigate the involvement of p38MAPK, the mitochondrial pathways of apoptosis (affected by p38MAPK and its co-conspirators), and explored early effects of HD on mitochondrial permeability.

3.5.   "Gene-Expression Profiles of Vesicant-Induced Skin Injury," Gerecke et al, USA

Gerecke et al (USA) observed that a likely target of SM's actions at the basement membrane was the glycoprotein, laminin-332, a product of three polypeptides that are separate gene products. The authors observed that one of the three polypeptides, laminin-gamma 2 polypetide, is preferentially upregulated in migrating skin cells (NHEKs) after SM injury. These results provided an understanding of cutaneous SM exposure and identified other potential diagnostic markers and targets for treating exposure to SM.

3.6. "Inhalation Toxicology of Sulphur Mustard: Dose-Ranging Study in a Porcine Model." Jugg et al, GBR

Jugg et al (GBR) investigated, in the pig, the pulmonary toxicity of SM, emphasizing the identification of early biomarkers of exposure. Anaesthetized, instrumented Large White pigs were exposed to air or inhaled HD (60,100, or 150 ug/kg^{-1}).
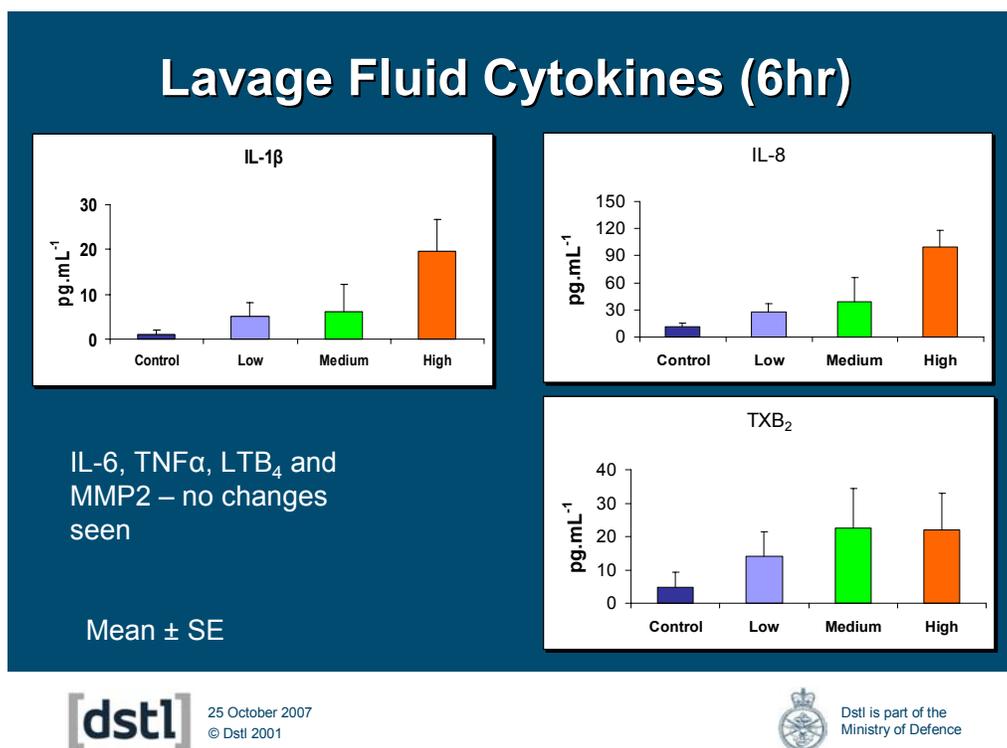

Figure 3. Dose-dependent changes in lavage fluid cytokines -- IL-1B, IL-8, and TXB$_2$ from Jugg et al, GBR.

Bronchealveolar lavage and histopathological exams formed the basis for these observations. See Figure 3. SM exposed animals showed dose-dependant effects in:

Oxygenation
Arterial pH
Lung wet weight/body weight
Cytokines IL-1B, IL-8
Lavage protein
Pathology (seen as similar to humans)

This study suggested possible biomarkers for pulmonary exposures.

3.7. Summary.

In medicine, a biomarker can be a substance whose detection indicates a particular disease state (for example, the presence of an antibody may indicate an infection). More specifically, a "biomarker" indicates a change in expression or state of a protein that correlates with the risk or progression of a disease, or with the susceptibility of the disease to a given treatment. Once a proposed biomarker has been validated, it can be used to diagnose disease risk, presence of disease in an individual, or to tailor treatments for the disease in an individual (choices of drug treatment or administration regimes). See Figure 2, Cassell et al, SWE). Pharmacogenomics can provide a tool to discover and begin to qualify biomarkers useful for these indications and is readily applicable to multiple species. One can begin and end with genes or focus on tissue-derived expression analysis of those genes that encode secreted proteins to discover potential biomarkers that can be monitored in body fluids. In Session II, Mustard and Nerve Agent Toxicology, the authors propose numerous newly identified biomarkers (e.g., cytokines IL-1B, IL-8) for validation, in some cases as therapeutic biomarkers, and in other cases as targets for therapeutic intervention (e.g., laminin-332).

4.0. DIAGNOSTICS OF OP EXPOSURE

- "Estimating Miotic, Severe, and Lethal Toxic Effects in Gottingen Minipigs Following Inhalation, Intravenous, and Subcutaneous Exposures to VX," Hulet et al, USA
- "Distortion Product OtoacousticEmissions as Potential Non-Invasive Predictors and Biomarkers of Soman-Induced Central Neurotoxicity," Job et al, FRA
- "Verification of Exposure to Organophosphates: Detection of an Unknown Cholinesterase Inhibitor," van der Schans et al, NLD
- "Red Blood Cell Acetylcholinesterase and Cholinesterase Status: Important Tools for the Physician in a Nerve Agent Scenario," Thiermann et al, DEU

4.1. "Estimating Miotic, Severe, and Lethal Toxic Effects in Gottingen Minipigs Following Inhalation, Intravenous, and Subcutaneous Exposures to VX," Hulet et al, USA.

Hulet et al (USA) exposed male Gottingen minipigs to inhalation, intravenous, or subcutaneous VX and observed them for pupillary constriction (miosis) or toxic signs resulting in a classification of lethal, severe or moderate intoxication. These VX findings were compared to earlier work in the same model using GB or GF. VX vapor was more potent than GB or GF when lethality was the endpoint. However, unlike GB or GF exposures, constriction of the pupils was not the definitive first noticeable effect after VX exposure and occurred at concentration near those that result in severe toxic signs or lethality.

4.2. "Distortion Product OtoacousticEmissions as Potential Non-Invasive Predictors and Biomarkers of Soman-Induced Central Neurotoxicity," Job et al, FRA.

Job et al (FRA) presented a novel non-invasive approach to monitor soman-induced CNS toxicity. Distortion-produced otoacoustic emissions (DPOAEs) were correlated with levels of soman intoxication and appear to serve as non-invasive biomarkers of CNS change. The DPOAEs were correlated with central ChE inhibition and pathological changes in the auditory sensory pathways.

4.3. "Verification of Exposure to Organophosphates: Detection of an Unknown Cholinesterase Inhibitor," van der Schans et al, NLD.

Van der Schans et al (NDL) discussed a generic method to verify exposure to nerve agents. The method was based on the conversion of the phosphyl moiety to conjugated BuChE and using common protein chemistry techniques to detect phosphylated proteins and a generic protein adduct measured by LC-MS/MS allowing for detection of a broad spectrum of OP agents that inhibited BuChE. Using this method, a broad spectrum of OP BuChE inhibitors can be detected (i.e., most of the OPCW schedule 1 ChE inhibitors) with reasonable sensitivity.

4.4. "Red Blood Cell Acetylcholinesterase and Cholinesterase Status: Important Tools for the Physician in a Nerve Agent Scenario," Thiermann et al, DEU.

Thiermann et al (DEU) described efforts to assess reduced red blood cell (RBC) acetylcholinesterase (AChE) rapidly and reliably. The field portable device Test-Mate [R] was compared to a lab-based automated analysis system (Tecan RMP [R]). Excellent correlations were observed between field portable and lab-based methods, and, as a result, the reduced RBC-AChE activity was associated, in a field trial using anaesthetized pigs, with intoxication due to a ChE inhibitor. In that trial, there were rapid increases in RBC-AChE activity following administration of a bolus of HI-6 on site, indicating the effectiveness of the oxime treatment.

4.5. Summary.

A biomarker can also be used to indicate exposure to various environmental substances in epidemiology and toxicology. In these cases described in Session III, the biomarker may be the external substance itself (e.g. asbestos particles or NNK from tobacco or regenerated OP), or a variant of the external substance processed by the body (a metabolite or perhaps an inhibited enzyme).

Collectively, these presentations in Sessions III provide an excellent overview on the state-of-the-art in chemical defense forensics. Based to a certain degree on the numerous allegations of CW exposure in the Gulf War, the data and methodologies described in these papers furthers our understanding of the dose-response relationships at exposure levels (in this case for the nerve agents) that just produce overt signs or symptoms, or are asymptomatic (Hulet et al, USA) or severely toxic (Thierman et al DEU). These studies have equal significance to either battlefield or terrorist use of CW agents, because in both situations the medical community will be called upon to confirm (or deny) that significant exposure to CW agent has occurred – and to make some inference regarding the clinical relevance of that exposure.

5.0. TOXINS.

- "Comparison of Deposition Patterns for Small and Large Particle Aerosolized Toxins and the Resulting Disease in Guinea Pigs and African Green Monkeys," Leffel et al, USA

- "The Production and Evaluation of Ricin Antitoxins," Holley et al, GBR
- "Retrospective Identification of Ricin in Animal Tissues Following Administration by Pulmonary and Oral Routes," Griffiths et al, GBR
- "Critical Role of the Sodium Hydrogen Exchanger in Maitotoxin-Induced Neuronal Cell Death in Cultured Rat Cortical Neurons," Wang et al, CAN

5.1. "Comparison of Deposition Patterns for Small and Large Particle Aerosolized Toxins and the Resulting Disease in Guinea Pigs and African Green Monkeys," Leffel et al, USA.

Leffel et al (USA) analyzed deposition of small (1 micron) and large (8 microns) particle aerosolized ricin in guinea pigs and African Green monkeys. In this model building effort they found that small particle intoxication was more severe than large particle intoxication, that ricin deposition was diminished by 24 and 48 hr, and in guinea pigs, there was significant large particle deposition at the 1 hr time point, but no small particle deposition. In the African Green monkey, similar results were found to the guinea pig in the small particle studies. However, they could not deliver enough toxin to calculate a large particle LD50. As with the Griffiths et al (GBR) paper below, by 48 hrs ricin had been transported to the alveolar macrophages.

5.2. "The Production and Evaluation of Ricin Antitoxins," Holley et al, GBR.

Holley et al (GBR) (presented by G. Griffiths) reported on the production and evaluation of anti-ricin antibodies raised in sheep following ricin administration. These included F(ab')2 and Fab' as well as polyclonal IgG purified from spleen. The protective efficacy of F(ab')2, Fab' and IgG were evaluated in mice at from 2 to 16 hrs after ricin challenge (systemic and inhalation). Both IgG and F(ab')2 were effective even when administered up to 16 hrs post challenge but Fab' was not. See Figure 4. The authors suggested that the work demonstrates the feasibility of producing an effective antitoxin for post-exposure use and indicated that further advanced development work on this product is likely.
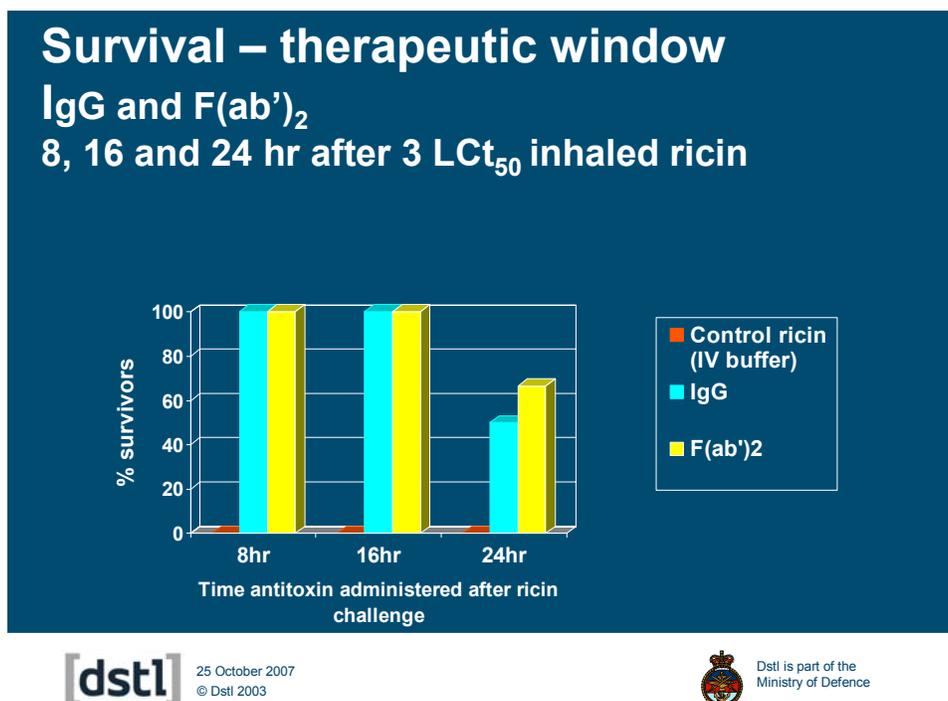
Figure 4. The efficacy of ricin antitoxins as therapeutic modalities – the "therapeutic window."

5.3. "Retrospective Identification of Ricin in Animal Tissues Following Administration by Pulmonary and Oral Routes," Griffiths et al, GBR.

Griffiths et al (GBR) discussed an effort to quantify ricin in selected tissues following ricin exposure. This assay would be desirable to detect and quantify ricin exposure, thus validating the requirement to administer antitoxin (a foreign protein, ovine IgG) during the "therapeutic window" (See Figure 4). Ricin was extracted from lung, blood, liver, and spleen with maximum yields in lung. These studies supported development of this amplified ELISA for ricin and pointed out the progression of ricin from surfaces of the lung into the lung tissue by 48 hrs.

5.4. "Critical Role of the Sodium Hydrogen Exchanger in Maitotoxin-Induced Neuronal Cell Death in Cultured Rat Cortical Neurons," Wang et al, CAN.

Wang et al (CAN) investigated the role of the cell membrane sodium hydrogen exchanger (NHE) in Maitotoxin-induced Ca^{2+} influx and subsequent cell death in cultured rat cortical neurons. Ca^{2+} influx was measured in a fluorimeter using fura-2 as a fluorescence indicator while total cell death was measured either with the *alamarBlue*[TM] cell viability assay or the ethidium bromide uptake assay. In cultured cortical neurons, Maitotoxin, a shellfish toxin, was found to increase both Ca^{2+} influx and cell death in a concentration dependent manner. Cells treated with maitotoxin showed a decrease in the phosphorylation of AKT, a serine/heroine protein kinas implicated in cell proliferation and survival. The actions of maitotoxin were dependent on extracellular Ca^{2+} and Na^+. Pretreatment of cells with the specific NHE inhibitor 5-(N-ethyl-N-isopropyl)-amiloride (EIPA, 1-10 µM) concentration dependently prevented maitotoxin-induced Ca^{2+} influx and cell death. EIPA also reversed the effect of maitotoxin on AKT phosphorylation. These findings indicate

that NHE is activated by maitotoxin in cultured cortical neurons. This activation of the sodium hydrogen exchanger results in a net increase in intracellular Na^+ concentration, membrane depolarization, activation of Ca^{2+} channels, and possibly massive Ca^{2+} influx and subsequent neurotoxicity.

5.5. Summary.

Toxins have several features that render them noteworthy as potential WMDs. These features include: significant human toxicity, past military interest, in the case of ricin, wide availability in ton quantities from castor seed meal, and increased attention from the world news media (giving them "shock value"). Session V focused on work toward practical medical solutions to one toxin (viz., ricin) and explores the in vitro biochemistry and pathophysiology of a lesser-studied toxin (viz., maitotoxin), pointing to the scope and diversity of the various national efforts.

6.0. OP MEDICAL COUNTERMEASURES

- "Comparison of Pretreatment Regimes Against Soman: In Vivo and In Vitro Analysis of Treatment Tolerability and Efficacy," Mikler et al, CAN
- "Prophylaxis Against Nerve Agent Toxicity: Physiological, Behavioral, and Neuroprotection Aspects of Current and Novel Treatments," Phillipens et al, NLD
- "Development of New Reactivators of Tabun-Inhibited Acetylcholinesterase and the Evaluation of Their Efficacy by In Vitro and In Vivo Methods, " Kassa et al, CZE
- "Central Cholinesterase Reactivation by Oximes Improves Survival and Terminates Seizures Following Nerve Agent Intoxication," Shih et al, USA
- "Pharmacological Screening of Anticonvulsants Against Nerve Agents: Limitations by Conventional Approach," Aas et al, NOR
- "Studies to Evaluate Novel Neuroprotectants in a Rat Model of Soman Exposure Reveal Episodes of Status Epilepticus and Spontaneous Recurrent Seizures even with Initial Oxime, Atropine, and Diazepam Therapeutics," Yourick et al, USA
- "The Effect of Anaesthetic and Oxygen Tension on the Toxicity of OP Agents," Sawyer et al, CAN (poster)
- "Protection Against Sarin-Induced Seizures in Rats by Direct Microinjection of Scopolamine, Midazolam, or MK-801," Skovira et al, USA (poster)
- "A New Approach for Future Pharmacologic Screening of Anticonvulsant Agents for Nerve Agent Poisoning," Myrher et al, NOR (poster)

6.1. Introduction.

The presentations on CW treatment and prophylaxis focused on a) development of a non-toxic pretreatment for nerve agent exposure that has no adverse side-effects, doesn't decrement performance, is easy to administer, and has a long biological half-life (e.g., Mikler et al (CAN)) or b) improving upon diazepam as a nerve-agent anticonvulsant through identification of neuroprotective or neuroresuscitive drugs to prevent seizure-related brain damage (Shih et al, USA or Aas et al NOR). Efforts to develop natural or recombinant enzyme bioscavengers for use as nerve-agent pretreatments continue to show promise and are discussed separately in the last section.

6.2. "Comparison of Pretreatment Regimes Against Soman: In Vivo and In Vitro Analysis of Treatment Tolerability and Efficacy," Mikler et al, CAN.

Mikler et al (CAN) presented a paper that exemplified the various programs' efforts to develop safe and effective pretreatments for their military forces. Mikler presented a comparison of Huperazine (HUP), Galantamine (GAL), and Caramiphen (CAR), a non-opioid antitussive pretreatment alone or in combination to control and physostigmine/scopolamine combination pretreatment in guinea pigs. He assessed tolerability for 7 days following pretreatment using observable signs of toxicity and body weight changes (in the absence of soman challenge). Mikler assessed efficacy (in vivo) by the combination's ability to prevent toxicity and death following a 1.5 x and 3.0 x LD_{50} soman challenge. He also assessed in vitro efficacy in an excised rat diaphragm/phrenic nerve preparation. In general they found that a HUP/CRM combination produced no observable toxicity and provided effective protection against soman. Conversely, a GAL/CRM combination provided protection against soman but produced some toxicity as a pretreatment which would limit its use. Finally, the authors reported that a HUP/CRM combination delays toxicity, even against a 3x LD_{50} challenge and that delay would provide time for countermeasures and additional treatment. The authors suggested that: (1) HUP/CRM should be considered as a future pre-treatment regimen, and (2) studies that include the use of post-exposure countermeasures are essential to determine the effectiveness of this pre-treatment.

6.3. "Prophylaxis Against Nerve Agent Toxicity: Physiological, Behavioral, and Neuroprotection Aspects of Current and Novel Treatments," Phillipens et al, NLD.

Phillipens et al (NLD) reported that a physostigmine (PHY)/scopolamine (SCO) combination has been put forth as a promising alternative to pyridostigmine. However, it has been suggested that other mechanisms contribute to the protection against OPs in addition to protecting the enzyme by reversible inhibition. Thus, PHY/SCO, and, in this case, procyclidine (PC), a drug with actions at both cholinergic and NMDA receptors, were tested as part of a PC/SCO combination and compared to PHY/SCO standard. Phillipens reported on studies in guinea pigs using behavioral, bio-physiological, neurophysiological and clinical assessments. In parallel studies, receptor binding and blood and brain AChE inhibition studies were performed. The PHY/SCO and PC/SCO combination showed very good protection in terms of survival and post-intoxication incapacitation. Presence of PC or SCO in the pretreatment prevented the appearance of full-blown seizures on the EEG. The authors suggested that protection is a result of temporary ChE inhibition (protection of the enzyme by reversible inhibition) and direct effects on both cholinergic and NMDA receptors. The PC/SCO combination has an added value in the protection against seizures, suggesting utility as a post-exposure therapy, an important advantage in field military medicine.

6.4. "Development of New Reactivators of Tabun-Inhibited Acetylcholinesterase and the Evaluation of Their Efficacy by In Vitro and In Vivo Methods, " Kassa et al, CZE.

Kassa et al (CZE) evaluated the enzyme reactivating effects of obidoxime and HI-6 as well as two newly developed oximes (K074, K075) in combination with atropine (ATR) in tabun- or cyclosarin-poisoned rats. In vivo reactivation studies demonstrated that only HI-6 could satisfactorily reactivate cyclosarin inhibited AChE but obidoxime, K074, and K075 were not effective. The neuroprotective effects of these oximes in combination with ATR were studied in rats poisoned with cyclosarin (80% of LD50 value). Neurotoxicity was assessed using a functional observation battery and automated activity monitoring at 24 hr and 7 days post-challenge. Again, HI-6/ATR was effective in decreasing neurotoxicity but not obidoxime, K074, or K075/ATR combinations. Thus it appears that the neuroprotective potency of oximes corresponded to their reactivating efficiency.

6.5. "Central Cholinesterase Reactivation by Oximes Improves Survival and Terminates Seizures Following Nerve Agent Intoxication," Shih et al, USA.

Shih et al (USA) compared the efficacy of tertiary oximes (diacetylmonoxime(DAM) and monoisonitrosoacetone (MINA)) versus quaternary oximes (2-PAM, HLo7, and MMB-4) in reactivating AChE inhibited by sarin in blood, brain, and peripheral tissues. Studies were performed in guinea pigs. Animals were challenged with 1 LD50 sarin and treated five min later with one of these oximes. At one hr post treatment blood and tissues were collected and ChE analysis was performed. ChE reactivation from quarternary oximes was marked in blood and peripheral tissues but zero in brain. However, treatment with tertiary oximes resulted in marked reactivation of AChE in brain, with little to no evidence of seizure and 100% survival.

6.6. "Pharmacological Screening of Anticonvulsants Against Nerve Agents: Limitations by Conventional Approach," Aas et al, NOR.

Aas et al (NOR) described a triple therapeutic regimen consisting of procyclidine, diazepam, and pentobarbital as effective in terminating soman-evoked seizures in rats when this regimen was administered as late as 30-40 min after seizure onset. A refinement of the triple therapeutic regimen resulted in a double regimen consisting of procyclidine (at a slightly higher dose) and propofol (50 mg/kg) effective at stopping soman-evoked seizures even when administered at 30-35 min post seizure onset (See Figure 5).
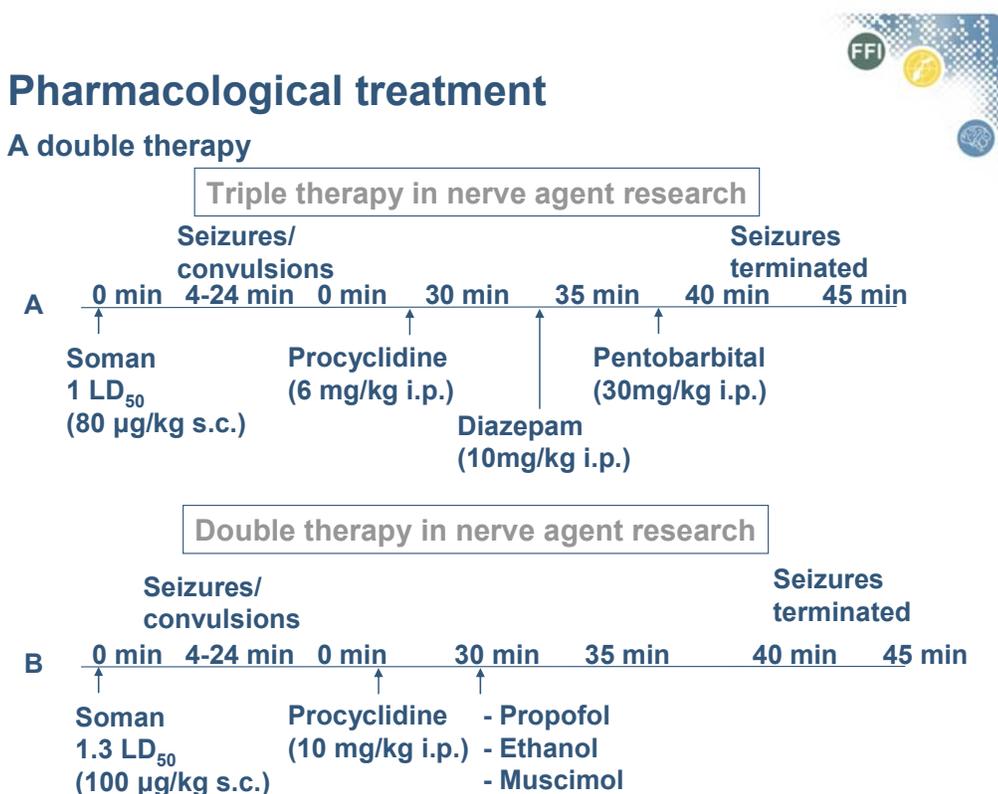

Figure 5. The concept of double therapy in nerve agent treatments, from Aas et al(NOR).

6.6. "Studies to Evaluate Novel Neuroprotectants in a Rat Model of Soman Exposure Reveal Episodes of Status Epilepticus and Spontaneous Recurrent Seizures even with Initial Oxime, Atropine, and Diazepam Therapeutics," Yourick et al, USA.

Yourick et al (USA) evaluated novel neuroprotectants, namely, NR2B-selective antagonists, serine racemase inhibitors, gabaergic enhancers and the like, providing protection against nerve-agent induced brain injury. They reported that up to 40% of rats experienced spontaneous recurrent seizures and additional episodes of status epilepticus (SE) for several days after soman exposure. Histopathological data revealed severe tissue damage in cortical and sub-cortical areas that was directly correlated to the number of secondary SE events following exposure. In terms of behavioral studies, the NR2B-selective antagonist ifendopril provided improved functional recovery. Other drugs reduced mortality, suggesting novel neuroprotection approaches, capable of reducing neuropathology, morbidity, and mortality, to the problem of nerve agent-induced brain injury.

6.7. Summary.

The presentations of the latest work in OP Medical Countermeasures emphasized the protection of the CNS compartment from OP intoxication. Pretreatments involving reversible inhibition of CNS enzymes or direct effect on cholinergic and/or NMDA receptors were presented in great detail. As a therapeutic approach, reactivators of centrally inhibited ChE were shown to be important for full recovery (Kassa et al, CZE and Shih et al, USA). Finally, improved anticonvulsants and neuroprotectants are being evaluated and demonstrating efficacy. Session VI clearly demonstrated the strength and diversity of the national programs addressing OP intoxication.

7.0. OPERATIONAL TOXICOLOGY

- "Some Mechanisms of Actions of Toxic Action of Irritants," Levchenko et al, UKR
- "Assessment of Respiratory and Renal Functions Among Gas Metal Arc welders and Their Relations with Chromium Exposure," Affify et al, EGY
- "Arsenic Trioxide: Estimation of Health risk on the Basis of Toxicological Indices," Juruli et al, GEO
- "Biomonitoring of Exposures to Permethrin Based on Adducts to Proteins," Noort et al, NLD
- "Environmental Hazard In Vitro Biomarker Discovery Tools," Halverson et al, USA
- "Risk Management of Exposure to Chemicals Under Operational Conditions (HFM-ET078)," Langenberg, NLD
- "A Toxicity Sensor System for Drinking Water Protection," van der Schalie et al, USA (poster)

7.1. Introduction.

As numerous deployments within the Alliance focus on peace-keeping operations, often in areas that have not kept abreast of internationally accepted industrial hygiene practices, there needs to be further emphasis placed upon medical surveillance measures. Fortunately, many of the methods and procedures necessary to ascertain or refute exposure to a toxicant are similar irrespective of the compound. Likewise, the question of "how clean is clean?" following environmental release of any toxic chemical (to include CW agents) is complicated as detection levels become ever lower. Simple extrapolation from known toxic levels to longer exposure intervals over longer durations will no longer suffice as Hulet et al (USA) advised us in Session III.

7.2. "Some Mechanisms of Actions of Toxic Action of Irritants," Levchenko et al, UKR.

Levchenko et al (UKR) reported that incapacitating agents, including sensory irritants, may be used in a variety of situations such as stopping or preventing reconnaissance-diversionary actions, law-enforcement, personal self-defense aims, crowd control and the like. In determining the risk/benefit for effective riot control agents, the basic requirement must be harmlessness for the health of their active components. Levchenko et al studied displays of resorptive action of the most widespread riot control agents: 2-chlorobenzylidene malononitrile (CBM; US nomenclature = CS) and dibenz[b,f]-1,4-oxazepine (DBO; US nomenclature = CR). Pathogenesis of intoxication of both irritants CBM and DBO is characterised with compromised tissue respiration which results in metabolic acidosis. Key findings reported were:

(1) The primary effects of CBM (CS) are associated with suppression of activity of cytochrome-c-oxidase. Conversely, DBO does not significantly affect cytochrome-c-oxidase activity.
(2) There was a dose-dependent disruption of internal environment and resultant metabolic acidosis with CBM (CS), but not for DBO (CR), and
(3) Poisoning by CBM (CS) may be treated by "Antidotes of cyan-ion."

7.3. "Assessment of Respiratory and Renal Functions Among Gas Metal Arc welders and Their Relations with Chromium Exposure," Affify et al, EGY.

Affify et al (EGY) reported on the assessment of chromium toxicity in metal arc welders exposed to chronic or subchronic chromium vapors. The primary target of chromium toxicity is the respiratory system, with high levels of blood and urine chromium (as well as B2 macroglobulin levels) correlated with decreased pulmonary function. Renal injury is to the proximal tubule.

7.4. "Arsenic Trioxide: Estimation of Health risk on the Basis of Toxicological Indices," Juruli et al, GEO.

Juruli et al (GEO) estimated the health risk of arsenic trioxide on the bases of its toxicokinetics indices in rats. Single doses (3, 30 and 100mg/kg) of arsenic trioxide were given to rats. The amount of As in blood and urine was determined at different times during the experiment and throughout the absorption and elimination phase. Arsenic appears to be eliminated in three phases. Arsenic excretion in urine does appear to be influenced by the initial administered dose. It is shown that 67.11% of administrated arsenic trioxide (100 mg/kg) is excreted by kidneys with $T_{0.5} = 3.6$ days; 73.9% is eliminated by urine with $T_{0.5} = 2.4$ days (30 mg/kg); and 68% is eliminated by urine with $T_{0.5} = 0.8$ days (3 mg/kg). Thus arsenic has a relatively long half-life.

7.5. "Biomonitoring of Exposures to Permethrin Based on Adducts to Proteins," Noort et al, NLD.

Noort et al (NLD) reported that permethrin can be absorbed through the skin, while oral and respiratory exposure can also occur. Soldiers can be exposed to rather high doses of permethrin by migration of the compound from clothing to the skin surface. Although permethrin is generally considered as a rather safe compound, a number of adverse effects have been reported subsequent to the first Gulf War. Biomonitoring of exposure to permethrin is usually performed by analysis of its urinary metabolite 3-phenoxybenzoic acid (3-PBA). However, chronic low-level exposures and cumulative exposures cannot be assessed by analyzing urinary biomarkers. Noort and colleagues are engaged in the development of a methodology to assess the cumulative internal dose of exposure to permethrin, which is based on the assumption that (reactive) glucuronide conjugates of the major permethrin metabolites 3-PBA and cis/trans-3-(2,2-dichlorovinyl)-2,2-

dimethylcyclopropane-1-carboxylic acid (Cl$_2$CA) will form persistent adducts to proteins, in analogy with the glucuronide conjugates of structurally related drugs. Within the framework of the current study, the 3-PBA and Cl$_2$CA glucuronide metabolites of permethrin have been synthesized. Subsequently, the reactivity of these metabolites with various amino acids, peptides and albumin has been studied. A number of distinct adducts could be identified by LC tandem mass spectrometry. Since in general albumin adducts have a rather long half life time (weeks), the identified adducts might form the basis for a novel biomonitoring methodology for exposure to permethrin.

7.6. "Environmental Hazard In Vitro Biomarker Discovery Tools," Halverson et al, USA

Halverson et al (USA) described efforts to identify useful biomarkers for a robust occupational environmental health surveillance program. He defined biomarkers as physiological or biochemical changes indicative of an exposure and/or effect of a toxicant. As part of the biomarker development effort two cell types, human peripheral blood mononuclear cells (PBMC) are being evaluated for their responses to environmental toxicant exposures. The PBMC are an ideal choice for an in vitro biomarker discovery tool since they are a component of an easily accessible biofluid (blood), which is an important biomarker requirement. In order to develop potential biomarkers of exposure and/or effect from these cellular models, we have utilized and integrated three distinct technologies, evaluated the subsequent data, and assessed the validity of the responses. The technologies utilized were quantitative proteomics using ion intensity, functional genomics with Affymetrix® microarrays, and an enzyme-linked immunosorbent assay (ELISA) which determines the proinflammatory cytokine and chemokine responses of the human PBMC. See Figure 6. The results of the cellular models exposure to toxicants such as trinitrotoluene (TNT) and dinitrobenzene (DNB) and the carbamate pesticide aldicarb were presented. Evaluation of the data generated from a 24 hour PBMC exposure to TNT and DNB identified proteins with at least a 1.5 fold quantitative difference when the exposed vs. unexposed cells were compared. Proteomic analysis, therefore, validated the biomarker of exposure. These data demonstrate the application of advanced proteomic analysis in validating potential biomarkers identified in functional genomic screening.
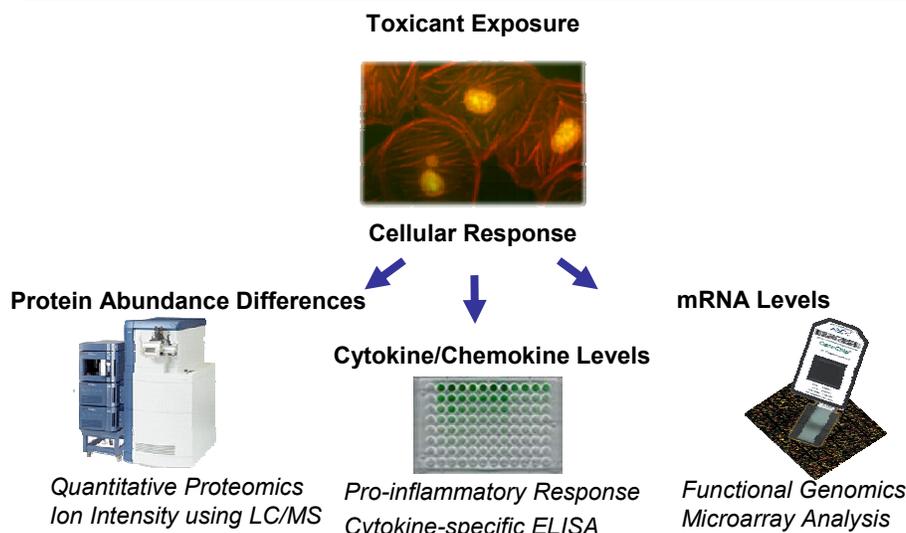
Figure 6. The application of advanced proteomic analysis in validating potential
biomarkers identified in functional genomic screening.

7.7. "Risk Management of Exposure to Chemicals Under Operational Conditions (HFM-ET078)," Langenberg, NLD

Langenberg (NLD) presented an update on ET-078: Risk Management of Exposure to Chemicals under Operational Conditions. The evolution of toxicology in support of operations was related to the evolution of threats since the end of Col War. The threat spectrum changed after Cold War in that large scale CW-attack seems unlikely, that small scale CW-attacks, low level exposure may occur, and that exposure to toxic industrial chemicals/materials (TICs/TIMs) may occur. ET-078 is shaped by two assumptions: (1) an increased level of safety and health care for military personnel under operational conditions will improve combat readiness and effectiveness, and therefore the probability of successful mission completion, and (2) an additional (non-operational) benefit is that the risk of post-deployment illness and disability, resulting from exposures during deployment, will be reduced.   The deliverable for ET-078 is technical report on the strategy for follow-on activities in this area. A well-attended session addressing this strategy question occurred on Tuesday evening and lasted 90 min.

7.8. Summary.

The notion of operational toxicologic risk assessment may be in its infancy. A comprehensive risk assessment involves a thorough understanding of the health effects of a given exposure, a validated biomarker, and a functional monitoring system. The thorough understanding of health effects is enhanced by recent developments in toxicogenomics. Toxicogenomics is a form of analysis by which the activity of a particular toxin or chemical substance on living tissue can be identified based upon a profiling of its known effects on

genetic material. Once viable, the technique should serve for toxicology and toxin-determination a role analogous to DNA-testing in the forensic identification of individuals. The paper by Halverson et al in this session and Ford et al in Session II reflect this approach. Thus, the papers in this session, and others in the symposium, provide glimpses as to where we are with respect to a number of known, common, environmental or occupational hazards that could be encountered in military operations. In the case of arsenic trioxide (Juruli et al, GEO) we find ourselves working with the hazard assessment. Conversely, in the case of permethrin we find ourselves working with the biomarker and the monitoring systems, as examples (Noort et al, NLD).

8.0. FUTURE OF MEDICAL COUNTERMEASURES

- "Low-Level Toxicolgy and the Human Toxicity Estimates," Reutter, USA
- "Intracellular Acidification: The Initiating Event in Sulfur Mustard Induced Cytotoxicity," Sawyer et al, (CAN)
- "Novel Mechanism to Explain Ketamine Efficacy Against Nerve-Agent Induced Seizures," Hilmas etal, (USA) (presented by T.Myers)
- "Strategies for the Development of Effective Broad-Spectrum Oximes," Worek et al, (DEU)
- "Stoichiometric and Catalytic Scavengers as a Protection Against Nerve Agent Toxicity," Lenz, (USA)
- "Methods of Advanced Wound Management for Care of Combined Traumatic and Chemical Warfare Agents," Graham et al, USA (poster)
- "Role of Antioxidant Enzymes in Regulating Wound healing in Vesicant-Induced Skin Injury," Gray et al, USA (poster)
- "Development and Validation of an Efficient Neurobehavioral and Neuropathological Model for Assessing Advanced Neuroprotective Compounds in Non-Human Primates," Myers et al, USA (poster)

8.1. "Low-Level Toxicolgy and the Human Toxicity Estimates," Reutter, USA

Reutter (USA) presented an overview of the multi-year Low-Level Toxicology Program (LLTP) in the US and its resultant revised toxicity estimates. In the late 1990's dose-response curves were incomplete and dealt with the high ends of the curves, and concentration-time profiles did not exist for most CWA. There were virtually no data points for inhalation exposures longer than 10 minutes, and most of the data were for 2-minute exposures. The LLTP was initiated to address these data gaps. The author pointed out that a wealth of toxicity data has been obtained, and, coupled with a meta-analysis of the historical toxicity data, there is considerably more confidence in many of the currently recommended human toxicity estimates.

8.2. "Intracellular Acidification: The Initiating Event in Sulfur Mustard Induced Cytotoxicity," Sawyer et al, (CAN)

Sawyer et al (CAN) reported that HD produced an immediate and significant concentration-dependent decline in cytosolic pH, and also inhibited the mechanisms responsible for restoring pH_i to physiological values. This concentration response closely paralleled the acidification of the extracellular buffer through HD hydrolysis. The toxicity of HD was found to be dependent on extracellular pH, with a greater than eight-fold increase in LD_{50} obtained in cultures treated with HD at pH 9.5, compared to those treated at pH 5.0. Assays of apoptotic cell death, including morphology, soluble DNA, caspase-3 activity and TUNEL also showed that as pH was

increased, much greater HD concentrations were required to cause cell death. The modest decline in HD half-life measured in buffers of increasing pH, did not account for the protective effects of basic pH. Sawyer et al proposed that HD causes an extracellular acidification through chemical hydrolysis and that this, in both a concentration and temporally related fashion, results in cytosolic acidification and an irreversible decline in pH_i that initiates the cascade of events that results in cell death.

8.3. "Novel Mechanism to Explain Ketamine Efficacy Against Nerve-Agent Induced Seizures," Hilmas etal, (USA) (presented by T.Myers)

Hilmas et al (USA) (presented by T. Myers) reported that KET and PCP provide neuroprotection through a powerful antagonism of excitatory neurotransmission, namely pre-synaptic inhibition of glutamate release and post-synaptic inhibition of N-methyl-D-aspartate (NMDA)-type glutamate receptors. In addition, galantamine (GAL) has been shown to protect against lethality, diaphragmatic muscle paralysis, and seizures as a pre- or post-exposure treatment against lethal NA challenge in guinea pigs. GAL can prevent NA-induced tetanic fade as a pre- or post-exposure treatment in ex vivo guinea pig phrenic nerve-hemidiaphragm preparations. Hilmas et al argued that GAL and KET or their analogs may represent a new generation of centrally active post-exposure therapeutics against NA challenge. These post-exposure treatments represent an improvement upon the current pretreatment strategy and may expand upon the current military, first responder, and hospital pharmaceutical cache to treat casualties of NA exposure.

8.4. "Strategies for the Development of Effective Broad-Spectrum Oximes," Worek et al, (DEU)

Worek et al (DEU) reported on recent studies on the reactivation of OP-inhibited human AChE by oximes. Almost fifty structurally different oximes designed to be broad spectrum (i.e., effective against many or all OP nerve agents) were tested on their ability to reactivate human AChE inhibited by GA, GF or VX. The results of this study indicate that various bispyridinium oximes were effective reactivators at high concentration but only few compounds were effective against all tested nerve agents. Thus, in order to meet this goal different strategies are conceivable: (a) Development of newly synthesized oximes with a sufficient efficacy against the range of threat agents or (b) a combined use of two or more oximes having a complementary spectrum. The ability of obidoxime and HI-6 combinations to reactivate human AChE inhibited by GA, GB, GF, VX and paraoxon was tested at human relevant oxime concentrations. The rationale for using this oxime combination was the fact that HI-6 is an effective reactivator of GB-, GF- and VX-inhibited AChE while obidoxime is effective in case of paraoxon and a reasonable reactivator of GA-inhibited AChE. Worek et al found that a combination of HI-6 and obidoxime (1) did not impair reactivation, compared to HI-6 or obidoxime alone, but (2) broadened the spectrum compared to the individual oximes. This pragmatic approach of two or more oximes having a complementary spectrum is favored for the improvement of oxime therapy.
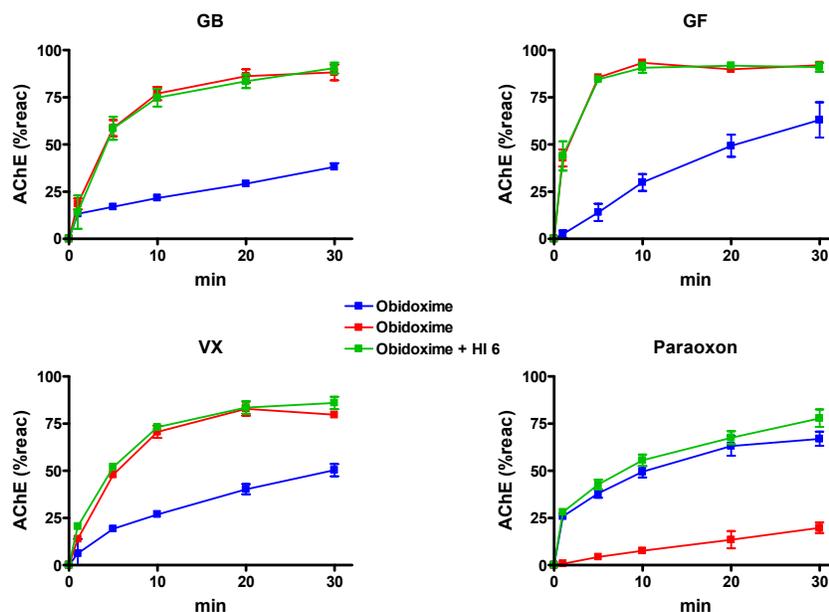
Figure 7. The "pragmatic approach" to development of broad spectrum oxime therapies. A combination
of HI-6 and obidoxime (1) did not impair reactivation, compared to HI-6 or obidoxime alone,
but (2) broadened the spectrum compared to the individual oximes.

8.5. "Stoichiometric and Catalytic Scavengers as a Protection Against Nerve Agent Toxicity," Lenz, (USA)

Lenz et al (USA) reported that proteins such as human plasma-derived butyrylcholinesterase (huBuChE) are able to neutralize the toxic effects of nerve agents *in vivo* and improve survival in guinea pigs and non-human primates and protect cognitive function in guinea pigs following exposure to a cumulative challenge of 5.5 LD_{50} of soman. Recently, a recombinant form of human butyrylcholinesterase (r-huBuChE), similar to plasma-derived huBuChE, has become available. Pharmacokinetic analysis of a polyethylene glycol coated (pegylated) form of r-huBuChE revealed rapid bioavailability and a half-life ($t_{1/2}$) which resembled that of plasma-derived huBuChE. When guinea pigs were administered pegylated r-huBuChE 18 h prior to exposure (sc) to 5.5 LD_{50} VX or soman, 100% survival was observed. These data supported the decision to select both plasma-derived and recombinant forms of BuChE for advanced development and transition to clinical trials in the US. Efforts are now focused on identification of a catalytic protein capable of hydrolyzing the standard threat nerve agents. Work to date has focused on paraoxonase-1 (PON1), a naturally occurring human serum enzyme. Using rational design several amino acids involved in substrate binding have been identified, and site-directed mutations have revealed that residue H115 plays an important role in determining substrate specificity. The effect of these mutations on the ability of PON1 to catalyze the hydrolysis of nerve agents was investigated and reported as noteworthy. Current studies are directed toward the identification of a PON1 mutant with enhanced catalytic activity (higher affinity and higher k $_{cat}$) for nerve agents as a next generation bioscavenger.

8.6. Summary.

Thus, Session VII demonstrated that oxime approaches and efforts to develop natural or recombinant enzyme bioscavengers for use as nerve-agent pretreatments continue to show promise. The obvious limitations imposed by simple stoichiometric agent binding appear to be overcome by recent data showing the efficacy of catalytic mutations capable of also breaking down the agent. The lack of behavioral impairment, as compared to pharmacological pretreatments is appealing.

9.0. CONCLUSIONS

Overall, the "Defense Against the Effects of Chemical and Toxic Hazards: Toxicology, Diagnosis and Medical Countermeasures" Symposium was a highly successful meeting. Participation by members of the Joint Medical Committee, as well as the emphasis placed on operational issues and applications by the Programme Committee, provided a unique perspective on the supporting chemical and biological defense technologies presented. The meeting was considerably more ecumenical than its predecessor in 2001, with 29 registered nations at this year's meeting vs. approximately 16 in 2001. Such cross-pollination is enormously beneficial to the member nations and their research and advanced development communities. Participation at this Symposium predominantly reflected those routinely engaged in chemical and biological defense issues as well as those charged with management of potential CB mass casualties. Future such symposia should seek to generate interest and participation from a much wider segment of the NATO community.

10. RECOMMENDATIONS

NATO/RTO/HFM should consider revisiting the topic of WMD defense and preparedness as the theme for a future Symposium in order to:

• Further integrate science, technology and operational and public health issues in keeping with policy guidance.

• Continue to push the biotechnology envelope in particular functional genomics, proteomics, toxicokinetics, and combinatorial drug approaches for both chemical and biological defense solutions.