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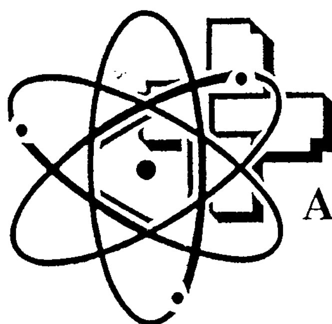
PROCEEDINGS OF THE

THE THIRD CHEMICAL AND BIOLOGICAL MEDICAL TREATMENT SYMPOSIUM

The CBMTS III

An Exploration of Present Capabilities and Future Requirements for Chemical and Biological Medical Treatment

The AC-Laboratorium Spiez
Spiez, Switzerland
7-12 May 2000



AC-Laboratory
Spiez

ASA

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Technical Agenda

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Co-Chair: David Moore
Sessions: 1, 2, 4, 5, 8, 9, 10, 11, 13, 15

Sector Biological Aspects
Chair: Barbara Price
Co-Chair: Ji-Sheng Chen
Sessions: 3, 6, 12, 14, 16

Note: All Papers / Posters as listed in remainder of schedule show only to which block they are assigned. Specific presentation order within the block will be determined by the Session Chair. Numbers within the parenthesis () are abstract numbers

Sunday 7 May 2000

- ____ - Participants arrive at the assigned hotels in Spiez or the Dormitory of the AC Zentrum Spiez
- **0930** - **Special Training Session**
- **1500** - **Special Training Session**
- **1600** - **Early registration and pick up of conference material**
- **1700** - **Buses pick up participants at the hotels in Spiez for registration**
- **1830** - **Meeting of Session chairs and co-chairs**
- **1900** - **Session chairs/co-chairs meet with their session presenters**
- **1930** - **Welcome party "Cheese and wine"**
- **2100** - **Busses return participants to hotel**

Monday 8 May 2000

- **0700** **Breakfast**
- **0745** - Busses promptly leave hotel area for AC-Laboratorium Spiez
- **0800** - **Registration and pick up of conference materials**
- **0830** - **The CBMTS III Opens**
  - Welcome address: **Dr Bernhard Brunner**, Director of the AC-Laboratorium Spiez
  - **Richard Price** CBMTS Organizer
  - **Dr. Brian Davey**, Chairman's address
- **0900** - **Introduction and Overview**
  - Scientific Review of CBMTS - Industry I (14) **Bokan, S.**
  - Operational Review of CBMTS - Industry I (16) **Orehovec, Z.**
  - Overview of the CBMTS Meetings **Davey, B.**
- **1000** - **Coffee**

Session 1: **Chair: Peter Eyer Co-Chair: Jiri Bajgar**

- **1030** _ **OP Treatment: State of the art, Practical treatment, Low Dose**
  - State of the Art Lecture: Organophosphorus Compounds and Esterases: Current Research Topics Concerning Toxicity of and Protection Against Organophosphates. (18) **Reiner, E.**
  - Role of serum cholinesterase activity in the treatment of organophosphate intoxication (71) **Ozyurt, G.**
  - The Pharmacokinetics in Healthy and OP Poisoned Subjects. (96) **Jovanovic, Du.**
  - Clinical Considerations which were Raised from the Experience Obtained in more than 300 Patients (96) **Joksovic, D.**
  - The Anticipated Organization of Medical Aid at the Battle Field (96) **Vojvodic, V.**
  - Effects of sodium bicarbonate in human organophosphate poisoning (34) **Balali-Mood, M.**
  - Simple method for more precise diagnosis and treatment of intoxication with nerve agents poisoning (22) **Bajgar, J.**
  - New aspects on the reactivation by oximes of organophosphate-inhibited human acetylcholinesterase in vitro (32) **Worek, F.**

- Assessing the Effects of Low Level Anticholinesterases (25) **Scott, L.**
- 1245 - **Lunch**

Session 2: **Chair: Elsa Reiner Co-Chair: Atilla Hincal**

- 1345 - _ **Poster: AnimalTreatmentStudies, Oximes, Cholinesterase Measurement**
  - Toxicological and Pharmacological Studies of HI-6 (3) **Dichovski, Ch.**
  - Efficacy of a combination of oximes and adamantanes against soman poisoning in mice (94) **Antonijevic, B.**
  - Search for New Neuroprotective Drugs Against Soman-Induced Central Neuropathology: Antioxidants (26) **Dorandeu, F.**
  - Memantine treatment improves antidotal efficacy of atropine, HI-6 and diazepam in rats poisoned with soman. (99) **Antonijevic, B.**
  - Quantum mechanical calculations in gas phase and polar medium and QSAR-analysis of a series 2- and 4-[hydroxi-imino)-methyl] – pyridinium derivatives. (4) **Dichovski, Ch.**
  - Study of Chemical Stability of HI-6 by Moleculeorbital Method (15) **Jukic, I.**
  - Acetylcholinesterase and butyrylcholinesterase activity measurements in human blood by the Ellman method: Procedure protocol and comparison with procedures suggested by other authors (19) **Simeon, V.**
- 1500 - **Coffee**

Session 3: **Chair: Murray Hamilton Co-Chair: Lotfalli Haghighi**

- 1530 - _ **Bioterrorism: National approach**
  - National Public Health Preparedness and Response to Bioterrorism (82) **Lillibridge, S.**
  - WMD Casualty Planning (91) **Spies, S.**
  - Canada's Response to biological and chemical incidents or terrorism (9) **Harwood, C.**
  - Bioterrorism and Australia - The Way Ahead (1) **Robertson, A.G.**
  - Compilation of a training curriculum for CB defense specialist advisers for the South African National Defence Force (58) **Erasmus, C.**
  - Non-Conventional Terrorism: Threat and Countermeasures (44) **Khan, L.A.**
- 1730 - **Bus to Hotel**
- 1730 - **Dinner**
- 1900 - **Bus to Hotel**

Tuesday 9 May 2000

- 0700 **Breakfast**
- 0745 - Busses promptly leave hotel area for AC-Laboratorium Spiez

Session 4: **Chair: Leah Scott Co-Chair: Florin Paul**

- 0800 - _ **OP Treatment Studies**
  - Physostigmine As A Pretreatment Against Soman-Intoxication Under Stress Circumstances (42) **Nieuwenhuizen, M.**
  - Effects of some reversible cholinesterase inhibitors on the reactivatability of Soman-inhibited human erythrocyte acetylcholinesterase in vitro. (12) **Zhang, Y.**
  - Efficacy of calcium channel blockers in soman-poisoned rats protected with atropine/HI-6/diazepam combination (95) **Dobric, S.**
  - Effects of Pyridostigmine (Pyr) and non Penetrative Head Injury of Neuromuscular Performance in the Rat.(20) **Moochhala, S.**
  - Structure and Efficiency of Carbamates as Drugs for Prophylaxis (53) **Tonkopii, V.**
- 0930 - **Coffee**
- 1000 - **ACLS Tour**
- 1230 - **Lunch**
- 1330 - **Blast Guard Demonstration**

Session 5: **Chair: Mahdi Balali-Mood Co-Chair: Cornelis Erasmus**

- 1430 - _ **Medical Treatment of Convulsions ; Mustard and Heavy Metal**
  - Imidazenil, a proposed drug for the treatment of convulsions in acute poisonings with organophosphates (46) **Kowalczyk, M.**

- Sulfur Mustard Induced Cytokine Changes in Different Cell Cultures (31) **Szinicz, L.**
- Prophylactic efficacy of Amifostine and its analogs against sulfur mustard intoxication. (33) **Jaiswal, D.**
- Iodine Protects Skin Against Mustard Gas (37) **Wormser, U.**
- Pharmacokinetic component of the mechanism of action of decorporators (47) **Mircioiu, C.**

- **1600** - **Photo**
- **1630** - **Coffee**

Session 6: **Chair: Leo Laughlin Co-Chair: Liaquat Ali Khan**

- **1700** - **_ Toxins**
  - "Hinge" Peptide Libraries As Inhibitors Of Botulinus Neurotoxin (8) **Hamilton, M.**
  - An Assessment of Toxins (11) **Chen, JiSheng**
  - History and Technological Evaluation of The U.S. Bio/Toxin Detection Program (81) **Laughlin, L.**
  - Biochemical and histological alterations in rats subacutely poisoned with T-2 toxin (97) **Jovanovic, Dj.**
  - Identifcation and Determination of Mycotoxins by HPLC in Liver (36) **Kalantari, H.**
- **1830** - **Bus to Hotel**
- **1830** - **Dinner**
- **2000** - **Bus to Hotel**

Wednesday 10 May 2000

- **0700** **Breakfast**
- **0745** - Busses promptly leave hotel area for AC-Laboratorium Spiez

Session 7: **Chair: Paul Russel Co-Chair: Sven-Åke Persson**

- **0800** - **_ B-Threat and Detection**
  - Criteria for Selection of Human, Animal and Plant Pathogens and Toxins to be Included in a List of Biological Agents and Toxins in Relation to the BTWC (13) **Bokan, S.**
  - Virus reproduction in macrophages: a common feature of viral hemorrhagic fevers (50) **Ryabchikova, E.**
  - Current Developments in Gene Technology: The Impact on Biological Weapons (67) **Schuetz, M.**
  - Conversion of Biotechnology (BW) Complex in Kazahstan: Scientific and Technical. The New Results (38) **Lepeshkin, G.**
  - Epidemiology and detection methods of the most important infectious diseases in the Republic of Macedonia (40) **Taleski, V.**
  - Epidemiological Approach of Biological Crisis Caused by Criminal Use of Bacillus Anthracis (48) **Paul, F.**
  - Novel Strategy Using Synthetic Nucleic Acids And Conventional Immunoassays For Biological Agent Detection (77) **Filer, S.**
- **1000** - **Coffee**

Session 8: **Chair: Gurayten Özyurt Co-Chair: Juli Tremblay-Lutter**

- **1030** - **_ Risk Assessment, Data Bank and Mass Casualty Management**
  - The Role of Drug and Poison Information Centers in Emergency Assistance and Professional / Public Education for CBW (70) **Hincal, F.**
  - Chemical Disaster Training For First Responders (79) **Hughart, J.**
  - Long-term Toxic Effects: Emerging New Challenges To Chemical Biological Risk Analysis And Medical Treatment Strategies (87) **Rao, V.**
  - Mass Casualty management: Poison Control Centers (80) **Kosnett, M.**
  - Mass Casualty Management in the Event of a Chemical Accident (39) **Mathenge, V.**

- Mass Causalty Management In Turkish Pharmaceutical Industry (69) **Hincal, A.**
- 1230 - **Lunch**
- 1330 - **Buses are leaving for Excursion**
- 1930 - **Bus to Hotel**
- 1930 - **Dinner**
- 2100 - **Bus to Hotel**

Thursday 11 May 2000

- **0700** **Breakfast**
- **0745** - Busses promptly leave hotel area for AC-Laboratorium Spiez

Session 9: **Chair: Richard Gordon Co-Chair: Urs Brodbeck**

- **0800** - _ **Riot control and Detection**
  - Toxicological Aspects of Events in Tbilisi on April 9, 1989 (29) **Juruli, M.**
  - Capsaicin in Pepperspray - Mode of Action (68) **Wicki, A.**
  - Scanning Flow Cytometry for kinetics study of antigen-antibody interaction on the cell surface (52) **Surovtsev, I.**
  - Biomedical Sampling: An OPCW Perspective (20) **Davey, B.**
  - Methods For Retrospective Detection Of Exposure To Toxic Scheduled Chemicals: An Overview (43) **Noort, D.**

Session 10: **Chair: Uri Wormser Co-Chair: Valerii Tonkopii**

- **0930** - _ **Cholinesterase Determination**
  - The determination of human erythrocyte acetylcholinesterase in a modified Ellman assay (30) **Eyer, P.**
  - Rapid, Quantitative, and Simultaneous Determination of AChE and BChE Levels in Unprocessed Whole Blood (76) **Feaster, S.**
  - Towards a Common Method for Measuring Cholinesterase Activity (64) **Portmann, R.**
  - Acetylcholinesterase and butyrylcholinesterase activity measurements in human blood by the Ellman method: Procedure protocol and comparison with procedures suggested by other authors (17) **Reiner, E.**
  - Evaluation of Methods for Interlaboratory Comparison Tests on Measurements of Cholinesterase Activity (62) **Brodbeck, U.**
- **1100** - **Coffee**

Session 11: **Chair: Devendra Jaiswal Co-Chair: Slavomir Rump**

- **1130** - _ **Decontamination**
  - Decontamination of casualties after exposure to harmful liquid chemicals (61) **Persson, S.A.**
  - Polyurethane Foam Linked Mammalian Cholinesterase for Decontamination and Detection of OP Nerve Agents (78) **Gordon, R.**
  - Mass Casualty Decon and Olympic Style NBC Response Factors and Solutions for CB Related Events (7) **Eaton, D.**
  - Reactive Skin Decontaminant Lotion (6) **O'Dell, P.**
  - Polyvalent technology for decontamination of heavy equipment (45) **Matousek, J.**
  - Gloves that fit and do not tear, a novel idea (10) **Laurie, E.**
- **1300** - **Lunch**

Session 12: **Chair: Elena Ryabchikova Co-Chair: Kim Lindsay**

- **1400** - _ **B-Agent Protection and Treatment**
  - Applications of an Effective Biocidal Technology for Medical Defense Purposes (85) **Moore, D.**
  - Smallpox, Monkey pox, and cowpox Viruses as Sources of New Therapeutic Drugs (51) **Shchelkunov, S. N.**
  - A Review of Infections among Iranian Combattants in the Iran-Iraq War (35) **Haghighi, L.**
  - Overview of Plague Vaccine (24) **Russell, P.**

- Biodefense Vaccines: Regulatory and Manufacturing Issues and Constraints (55) **Melling, J.**

- 1530 - **Coffee**

Session 13: **Chair: Keith Vesely Co Chair: Michael McMillan**

- 1600 - **Poster 2 Chemical Poisoning, Tratment, Warning, Decontamination**
  - Inhalation intoxication with sarin: relationship between sarin dose and cholinesterase inhibition in blood (21) **Bajgar, J.**
  - Long-term effects of low level inhalation exposure of rats to sarin (23) **Kassa, J.**
  - The Spanish Armed Forces Autoinjector for Nerve Agent Antidote (60) **Pita, R.**
  - Monitoring Of Potential Health Effects Of Nerve Agent Destruction In Shchuch'ye, Kurgan Oblast (South Ural), Russian Federation (65) **Robinson, S.**
  - Minimizing the Ecological Consequences of Chemical Agent Destruction (88) **Chaika,Y.**
  - Riot control agents and the CWC and potential CW-threats in the 21st century (54) **Matousek, J.**
  - Creation of modern systems of warning of population and personnel during emergency at the sites of chemical weapons storage and destruction in Russia (49) **Petrov, V.**
  - On The Old Military "Heritage" In Georgia (28) **Juruli, M.**
  - Cholinesterases, Oximes, and OP Hydrolases in Tandem can Hydrolyze Organophosphates (84) **Doctor, B.**

Session 14: **Chair:Lisa Rotz Co Chair: Maarten Nieuwenhuizen**

- 1715 - _ **Poster 3 B-Agents and Terrorism**
  - The Swiss Approach to Conteract Chemical Terrorism. (63) **Huber, U.**
  - Detection and early warning for terrorist CW-attacks (98) **Matousek, J.**
  - Infectious diseases trends during 3 years after the war in Croatia (1996-1998) (5) **Gotovac, P.**
  - Risk assessment of Agrochemicals to be used by Terrorists (59) **Yousefi, V.**
  - Immunodetection Of Biological Agents By Repertoire Cloning (66) **Schuerch, N.**
  - Buforin I, a natural peptide, inhibits Botulinum toxin B activity ??? (74) **Doctor, B.**
  - Efficacy of various corticosteroid regimens in treatment of acute T-2 toxin poisoning in rats. (100) **Jovanovic, Dj.**
- 1830 - **Bus to Hotel**
- 1930 - **Bus Pick up**
- 2000 - **Symposium Dinner**
- 2230 - **Bus to Hotel**

Friday 12 May 2000

- 0700 **Breakfast**
- 0745 - Busses promptly leave hotel area for AC-Laboratorium Spiez

Session 15: **Chair: Bhupandra Doctor Co-Chair: Yongxiang Zhang**

- 0800 - _ **Bioscavenger**
  - Kinetics of Nerve Agent Hydrolysis by a Human OPAA Hydrolase (72) **Broomfield, C.**
  - Antagonism of Organophosphorous Lethality with Sterically Stabilized Liposomes (Sl) Containing Recombinant Organophosphorus Acid Hydrolase (OPH) (93) **Way, J.**
  - Recombinant Cholinesterases: Developing an Ideal Bioscavenger for Protection against Organophosphate Nerve Agents (90) **Saxena, A.**
  - Scavenger Protection Against Organophosphorus Agents By Cholinesterases (75) **Doctor, B.**
  - Wide Range Application of Alteromonas Prolidase for Decontamination of G-Type Chemical Nerve Agents (73) **Cheng, T-C.**
  - Diisopropylfluorophosphate (DFP) Antagonism by Recombinant Organophosphorus Acid Anhydrolase (OPAA) Encapsulated within Sterically Stabilized Liposomes (SL) (86) **Petrikovoics, I.**
- 0945 - **Coffee**

Session 16: **Chair: Ladislaw Szinicz Co-Chair: Andrew Robertson**

- 1015 - _ **Terror**
  - Swiss Protection Equipment Training and Availability (57) **Centonze, A.**

- Communication in the Heat of a Crisis - The Crucial Element for Effective Crisis Decision Making (2) **Santana, G.**
- Prioritizing Potential Biological Terrorism Agents for Public Health Preparedness in the United States: Overview of Evaluation Process and Identified Agents (89) **Rotz, L.**
- The Development of Both Laboratory Protocols for Identifying Bioterrorist Threat Agents and A National Network for Information Dissemination to State Public Health Laboratories In the United States (83) **Lindsey, K.Q.**
- Risk Assessment of Bioterrorism (56) **Baumberger, Ch.**
- Electronic Syndromic Surveillance for Bioterrorism at the World Trade Organization Ministerial Conference in Seattle, Washington, 2000 (92) **Treadwell, T.**

- **1200** - **Lunch**
- **1300** - **Session Report**
- **1400** - **Coffee**
- **1430** - **Future and Closing**
- **1530** - **Bus to Hotel**
- **1800** - **Dinner**
- **1900** - **Bus to Hotel**

Saturday 13 May 2000

- **0700** **Breakfast**

SUMMARY OF SESSIONS

SESSION 1
by Peter Eyer

E. Reiner gave an overview on the current research topics concerned with esterases and OP compounds. She addressed basic and applied sciences as well with regard to the influence of allosteric modulators on inhibition. ageing and reactivation of serine esterases.
Pertinent questions:
Is there a "backdoor" for products to leave the active site of AChE in the deep bottom of the gorge? Which amino acid residues contribute to stabilization of the Michaelis complex between enzyme and OP and between inhibited enzyme and reactivator? Which molecular features influence the ageing process? Which factors influence the fate of the phosphyloximes? How is the catalytic machinery of the phosphotriesterases composed? Waiting for the 3-D structure! What are the sequences of reactions/events following ageing of NTE and resulting in long axon degeneration?

M. Balali-Mood presented results of a study aimed at investigating the effects of extra sodium bicarbonate infusion (beyond correction of acidosis) resulting in weak alkalinization (pH 7.47) on the outcome of OP-poisoned patients which all received atropine therapy (matched control group).
While there was the impression of some benefits of bicarbonate therapy (1 vs 3 deaths), statistically significant differences were not established yet. Due to the low costs of bicarbonate therapy the study will be extended to include additional patients with even more intensive alkalinization (aim pH 7.5 to 7.55).

J. Bajgar presented a study anticipated to identify the underlying nerve agent in the case of erythrocyte-AChE inhibition.
The method makes use of the different ageing half-lives of phosphonylated AChE as revealed by the reactivatability *in vitro*, e.g. in the presence of 50 _M trimedoxime. Since the method determines the reactivation in whole blood. reactivatability may be underestimated due to phosphonyloxime formation.

F. Worek presented results on reactivation experiments *in vitro* by oximes of sarin-inhibited blood esterases.
It became clear that any effective reactivation by oximes of plasma BChE generates additional phosphonyloximes that may re-inhibit AchE. Hence, a good reactivator of AchE should be quite ineffective towards BChE. Moreover. it appears that, in contrast to other 2-aldoximes such as HI 6, 2-PAM forms quite stable phosphyloximes. Thus effective reactivation by 2-PAM of inhibited AChE at physiological (high) concentrations may be thwarted.

L. Scott demonstrated a highly advanced approach to follow subtle behavioural and electrophysiological changes upon low-dose exposure towards OPs.
It appears that sign-free exposure of non-human primates towards sarin was without any significant effects on EEG or cognitive functions. The model seems to be suitable for studying potential long term effects of other compounds of interest, and additional biologically relevant indices can be incorporated.

POSTER SESSION 1
by Elsa Reiner
Atilla Henical

This session included animal treatment studies of oximes. Three posters (Abstracts 3,4 and 15) were not displayed because the authors cancelled their participation. The authors in this session gave brief platform presentations of their posters while the discussions took place in the poster area. This part of the session was extremely lively. very stimulating for further work and a most obvious confirmation that poster presentations should be organized in that way.

SESSION 3. BIOTERRORISM: A NATIONAL APPROACH
Chair: Dr. Murray Hamilton
Co-Chair: Dr. Lotfalli Haghighi

Col. Khan from Pakistan opened the session with a presentation detailing the characteristics of modern terrorists as they have moved away from large organizations toward smaller but more focussed groups. He suggested that nuclear biological and chemical attacks were within the wherewithal of these technically rather sophisticated organzations. The response of the national authorities should be organized around ensuring that there exists in each country sufficient stockpiles of vaccines, antidotes and therapeutic drugs to handle at least one "worst case" scenario.

During the question period Col Khan was asked if he really thought a nuclear attack was within the abilities of terrorists. He allowed that although it may not be likely, but it was still possible albeit with difficulty.

Following Col Khan's presentation, Dr. Cornelis Ersamus provided an overview of a curriculum aimed at teaching individuals in the basics of responding to CB attacks. Two factors that Dr. Erasmus stressed were: academic level of the training, which he determined was best set at the advanced high school level (Grade 12). The second ingredient that Dr. Erasmus indicated was very important is an understanding, at least in the rudimentary sense, of organic chemistry. The curriculum he developed contains about 12 hours of introductory organic chemistry, which greatly increased the trainee's appreciation of the problems associated with responding to a CB incident. The curriculum developed into a three week (8 x 40 min /day 5 days a week for 20 days) course which was trialed successfully in October of 1999. Future developments will include expanding the integration of biological agents within the different sections of the curriculum and perhaps offering this course in cooperation or collaboration of other countries, particularly those in the region of South Africa.

Dr. Colin Harwood then presented the actions and responsibilities that could be expected from of Health Canada (HC). Dr. Harwood is essentially designing the program at HC *de novo* and stressed that the CB aspects are only now beginning with the establishment of a committee of experts from the medical community, but outside the NBC field, to advise on appropriate response strategies. He stressed that HCs response would consist of two major factors: advice and training to the provincial and local first responders/planners and provision of medical supplies from first aid triage packages right up to 200 bed hospitals "in-a-box". One further area of concern, as expressed in Col Khan's talk, was the uninterrupted supply of adequate and the possible (probable) lack of appropriate drug/vaccine stockpiles. This, it appears, is a concern that is rapidly achieving a much higher profile that earlier.

Dr. Tracey Treadwell graciously agreed to substitute for Dr. Scott Lillibridge and report on the plans within CDC to prepare for potential CB attacks. Dr. Treadwell described how the budget for national preparedness of ~121M was distributed among various state and municipal governments. The program was essentially divided into two areas: drug and medicine stockpiles and devices. The monies for this program are well distributed among the states and the programs are underway. The imminent WMD trial "top-off" should go a long way to validating the national preparadness in the U.S.

The next presentation came from Mr. Steven Spies of EAI corporation, a company that trains WMD counter-terrorism and develops plans fro responding to CB threats. He listed some potential difficulties terrorists might encounter in attempting to mount a CB attack including: 1. Variations in biological strain potency/infectivity; 2. Not all WMD are easy to obtain; 3. Transport, weaponization and storage are problematic; 4. Effective dissemination is difficult; <u>but</u> 5. Some common chemicals are also WMDs. He then listed the 4 triage categories that will be encountered and then showed projections that could be expected from a reasonably successful attack (50% dissemination efficiency: ~1-15% require ACLS; ~5-25% are non-emergency but will require treatment; 3. ~50-90% will not require medical intervention; and 4. ~ about 1-5% are dead or will die). Planning around and predicting these types of scenarios is the training goal for first responders.

Dr. Andrew Robertson finished the session with an overview of the Australian readiness and response planning. He made the point early on that Australia has had its fair share of terrorist incidences and indeed needed to update its SOPs. An important observation is that, although the number of terrorist incidences has declined in recent years (from a high of ~600 in 1988 to <200 in 1998) the lethality (some might say effectiveness) of the individual attacks has increased. The Australian model is based on information gathering, counter-acquisition, passive protection and consequence management. The fact that Dr. Robertson's presentation was in fact an excellent summary of the previous 5 talks, clearly indicated that all countries are concerned and thinking in the same manner. Whether this is a good thing or not remains to be seen.

SESSION 4: OP TREATMENT STUDIES

Chair: Leah Scott Co-chair: Florin Paul

1. I. Phillipens: presented a summary of a number of guinea pig studies which investigated whether a range of stressors influenced a) the effects of pretreatment involving physostigmine and b) the effectiveness of such pretreatment in protecting against soman - induced clinical signs and lethality. Cortisol levels were monitored during

the studies and although the biochemical analysis of ChE inhibitor are incomplete, it was suggested that stressors induced adverse behavioral effects in pretreated animals and that protective efficacy was compromised. A number of possible mechanisms were proposed and discussed. It was clear that further studies are required to elucidate the impact of stress on medical countermeasures for nerve agent poisoning.

2. W. Zhou: described in vitro studies involving cholinolytic drugs and oximes. Five compounds with different profiles of human erythrocyte acetylcholinesterase inhibition were investigated. Unlike the oximes, toxogonin and HI-6, these compounds did not, by themselves reactivate acetylcholinesterase inhibited by soman. The results confirmed that, if added at the start of aging, reversible cholinestherase inhibitors increased the portion of acetylcholinesterase reactivatible by the oximes. There was discussion about the mechanisms concerned and related work.

3. V. Tonkopii: discussed the effectiveness of a series of familiar and less familiar carbamates as pretreatment for nerve agent poisoning in vivo. He indicated the historical background and a number of key issues including the importance of selection of animal species for such studies and the complex mix of factors which influence the effectiveness of individual carbamates. It was noted that for example some compounds could inadvertly enhance nerve agent toxicity. The potential mechanism of action was discussed at length. There were particular concerns about the time scales and juxtaposition of reversible and irreversible acetylcholinesterase inhibition.

SESSION 5: MEDICAL TREATMENT OF CONVULSIONS - MUSTARD AND HEAVY METALS

Chair: Prof. Mahdi Balali-Mood

Co-chair: Dr Cornelis M. Erasmus

Imidazenil, a Proposed Drug for the Treatment of Convulsions in Acute Poisonings with Soman (46) presented by Prof S. Rump (on behalf of M. Cowalczyk).

Prof. Rump first pointed out the side effects of benzodiazepines, and in particular, diazepam as anti-convulsant, and then introduced imidazenil as the ideal drug for the management of OP-induced convulsions. The anti-convulsant effects of imidazenil were studied on mice, using a convulsometer and on rats, using electroencephalographic (EEG) techniques.The antilethal efficacy of imidazenil was examined on mice with the standard therapy of atropine and HI - 6, by means of LD_{50} determinations of soman. It was concluded that imidazenil in a dose of 2 mg/kg blocked soman induced convulsions, and increased the anti-lethal effectiveness of atropine and HI - 6 in poisonings of mice with soman.

Sulphur Mustard Induced Cytokine Changes in Different Cell Cultures (31) presented by Dr L. Szinics

Dr Szinics pointed out that although the role of inflammatory cytokine released after chemical injury is well known, the respective effects of sulphur mustard (SM) are not well known. Thus the release of cytokines during the first 8 hours after SM exposure was investigated in human leukocytes and in two permanent, related cell lines (SCL II and HaCaT). The data indicate HaCaT cells to be a more appropriate model for the study of SM effects in keratinocytes compared to SCL II cells.

Prophylactic Efficacy of Amifostine and its Analogs Against Sulphur Mustard Intoxication (33) presented by Dr D. Jaiswal.

Dr Jaiswal pointed out that, despite research over the last several decades, no satisfactory prophylactic for the treatment regimen has evolved for sulphur mustard. He introduced Amifostine and its analogs as prophylactic agents for SM poisoning. The study was performed in mice by intraperitoneal administration of the compounds 30 minutes prior to the application of SM. It was found that Amifostine and one of its analogs, DRDE-07, gave significant protection.

Iodine Protects Skin Against Mustard Gas (37) presented by Dr U. Wormser

Dr Wormser carried out a prolonged study on the therapeutic effects of iodine at 15, 30, 45, and 60 minutes after SM exposure, based on clinical and pathological investigations on guinea pig skin. Gross pathology analysis showed a strong protective effect at intervals of 15 and 30 minutes, and to a lesser extent at 45 and 60 minutes. He concluded that the iodine preparation is a potential antidote against skin lesions induced by SM. The iodine preparation was not explained.

Pharmacokinetic Component of the Mechanism of Action of Decorporators (47) presented by Dr C. Mircioiu

Dr Mircioiu presented the pharmacokinetics of calcium edetate after single i.v., i.m. and oral administration, and after multiple i.m. administration in healthy volunteers. The pharmacokinetic model appeared to be

bicompartmental, in spite of the fact that the ion could not leave the central compartment. The study was then extended to total edetates, including Ca, Zn and Cu complexes. It was concluded that the system was generally unstable owing to high ionic strength, but was potentially more biodisposable, as was revealed by an increased skin transfer face to solutions of the same chelators.

SESSION 6: TOXINS
Dr. L. L. Laughlin

This short session included only three papers.

I discussed selected US BW Agent detection technologies from an historical perspective; these included spectroscopic methods, staining techniques, particulate methods including fluorescent and radioactive antibodies. chemiluminescence and Mass Spectrometry. The initial problems identified in the methods discussed still persist: sensitivity, time for detection/identification, and specificity of the methodology.

Professor Chen from China provided an assessment of toxins that clearly indicated that that there are far more toxins of concern than are currently listed in the Chemical or Biological & Toxins Weapons Conventions. He described a number of factors to assess the threat from toxins: the problems associated with medical treatments, the inherent toxicities of the materials, their molecular weights and chemical structures, and the origins of the various toxins. including both peptide and non-peptide toxins. All of these have a significant impact on medical treatment of intoxications.

Dr. Hamilton, from Canada, explained how botulinum toxins inhibited acetylcholine release by cleaving presynaptic membrane proteins necessary for exocytosis of the neurotransmitter acetylcholine. Then he described his group's work using a model peptide that contains a cleavage site as a substrate for peptide library screening. The library was prepared by combinatorial synthesis and the peptides contained a novel "hinge" section that allowed the peptide sequence to fold back on itself for better fit into the binding site. The peptides were screened to identify inhibitors to botulinum toxin. Specific libraries that contained a repeating sequence (probably the binding recognition site) did inhibit botulinum toxin. The ultimate aim is to develop and identify from combinatorial synthesis novel proteins that can be used to treat botulinum intoxication.

SESSION 7: B-THREAT AND DETECTION
Session Chair; Paul Russell Session Co-Chair: Sven-Ake Persson
Speakers: Slavko Bokan: Criteria for Selection of Human, Animal and Plant Pathogens and Toxins to be Included in a List of Biological Agents and Toxins in Relation to the BTWC
Elena Ryabchikova: Virus reproduction in Macrophages: A Common Feature of Viral Haemorrhagic Fevers
Martin Schutz: Current Developments in Gene technology: The Impact on Biological Weapons
Vaso Taleski: Epidemiology and Detection Methods of the Most Important Infectious Diseases in the Republic of Macedonia
Florin Paul: Epidemiological Approach to Biological Crisis Caused by Criminal Use of *Bacillus anthracis* The main theme of the session was identifying infectious diseases and toxins as candidate biological warfare agents and techniques to assist diagnosis of both natural disease outbreaks and biological warfare agent otbreaks.
In the first instance criteria based on biological and physical properties of pathogens and toxins such as infectivity. lethality, transmissability, disseminability and weaponisation were discussed as tools for identifying candidate biological warfare agents against human, animal and plant targets. A scoring mechanism was used to create a threat list which largely concurred with agents listed in the BTWC. A question raised the issue of whether arthropod vectors should be included.
In identifying disease outbreaks, some examples of recent emerging and re-emerging infections were highlighted with particular reference to Macedonia and the recent refugee crisis arising from the Balkan crisis. The use of rapid PCR and light cycler were mentioned as a rapid means of diagnosing a disease outbreak. The use of genetic techniques for rapid identification and epidemiology was discussed using Swiss studies of army training areas as an example. The dual use of these techniques was highlighted and the fact that recombinant gene research had increased dramatically over the last 15 years. Although most research efforts are laudable, the less ethical use of the technology and information derived from human genome programme should be considered. The best means of preventing such research would be through the BWTC and honesty in such fora as the CBMTS meetings.

Direct epidemiological studies were discussed as a means of identifying a disease outbreak due to the dissemination of a biological weapon, using anthrax as an example. The epidemiological curve, with casualty numbers and fatalities were presented and the current medical countermeasures to anthrax were shown.
The role of macrophages in viral haemorrhagic fevers(VHFs) was presented, indicating that most viruses causing VHF are able to multiply within these cells. Viral replication in macrophages is seen in both animal models and humans, but some animal models, such as the guinea pig and monkey species, do not show haemorrhagic rash. Haemorrhagic rash, and bleeding, however, were seen in the baboon model.

SESSION 8: RISK ASSESMENT, DATA BANK AND MASS CASUALTY MANAGEMENT
Chair: Gurayten Ozyurt Co Chair: JulieTremblay- Lutter
The Role of Drug and Poison Information Center in Emergency Assistance and Professional/ Public Education for CBW
Presenter: F HINCAL
Drug and Poison information Centers may well be linkage of medical institutions, military groups, police forces. civil defense personnel and public transportation system in the mass casualty management.
Chemical Disaster Training for first Responder
Presenter: JL HUGHART
The developing database consists of information about chemical disaster to be encountered by first responders: military men or women, disaster assessment teams, police officers, firemen and emergency medical technicians was introduced. It has key words as DISASTER, ASSESSMENT and ASSISTANCE.
Long Term Toxic Effects: Emerging New Challenges to Chemical Biological Risk Analysis and Medical Treatment Strategies
Presenter: V RAO
The understanding the long-term toxic effects of environmental toxicants and example data sets from DYNCORP Project covering civilian and military risk management are presented
The Role of Poison Control Centers and Medical Toxicologists in Responding to Chemical and Biological Incidents
Presenter: MJ KOSNET
In the event ofCB incident, Poison Centers can provide the key services: a) Centralized communication to hospital emergency department b) assistance of antidote and related emergency medical supplies, c) communication of information to the general public either through the media or coming telephone calls.
Mass Casualty Management in the event of Chemical Accident
Presenter: V MATHENGE"
Industrial accidents are becoming a common feature in the process of industrial development. The proactive action that would prevent the hazards of these factories brought by natural and human induction, was introduced.
Mass Casualty Management in Turkish Pharmaceutical Industry
Presenter: A Hincal
The Mass Casualty Management programs and preparation for Pharmaceutical Industry which is mainly located in Istanbul, Turkey, and their risk management approaches were introduced.

SESSION 9 RIOT CONTROL AND DETECTION
Chair: Richard Gordon
Co-Chair: Urs Brodbeck

Speaker 1 B. Davey "Biomedical Sampling: An OPCW Perspective
Dr. Davey (OPCW) pointed out that the convention states that biomedical sampling is possible and permitted, but that little is currently being done. This means that the accreditation for the labs for environmental samples is established, but has not to date been done for labs performing biomedical sampling. Biomedical sampling should go to labs according to the current procedure for other samples (eg environmental, including random selection of 3/12 labs). On a more positive note, OPCW associated labs will be prepared for reception and handling of biomedical samples in September of 2000.

Speaker 2 D. Noort " Methods for Retrospective Detection of exposure to Toxic Scheduled Chemicals: an overview"
Dr. Noort demonstrated their laboratory reason for diagnosis of adduct agents for retrospect detection, rather than metabolites. For instance, after sulfur mustard exposure, the urine metabolites last for only a few days. But adducts,

on the other hand, are persistent for 60-80 days (up to 94 days with higher doses). For nerve agents, 30% BChE inhibition is restored in 20 days, so the cholinesterase assay is useless after that time period. In contrast, fluoride induced reactivation (.25M, 15 min) with OP inhibited samples can give an indication of the OP 60 days later after the exposure due to adducts with the enzyme and possibly other serum proteins.
A lively discussion resulted. Some questions and comments posed were that there is a large instrument requirement. i.e., a mass/spec. The KF reversal procedure would not be applicable to soman adducts since they age. There was agreement that the best applicable methods should be established and standardized upon by the different groups who would perform adduct analysis.

Speaker 3 M. Juruli. "Toxicological Aspects of Events in Tbilisi on April 9, 1989"
This paper was presented for authors who were not in attendance. Dr. Juruli concluded that due to the neurological activity and pathology indications of severe to moderately affected individuals during the Tbilisi incident, these people should be more closely followed in the future.

All questions were referred to the original authors by mail.

Speaker 4 I. Surovtsev "Scanning flow cytometry for kinetic study of antigen-antibody interaction on the cell surface"
Ivan described the scanning flow cytometry system and contrasted the system with conventional flow cytometry. He also presented his mathematical model for the analysis of Ab/Ag and cell surface interactions. He demonstrated that more information man be obtained by evaluating the interactions kinetically rather than at steady state. He showed traces of several systems, including two strains of E. coli. There was a lot of discussion on his last slide where he showed probability on the y-axis. The probability numbers were small ($x10^{-7}$), and it was difficult to correlate the values with a physical parameter (binding constants between antigen and antibody on the surface of cells).

Speaker 5 A. Wicki "Capsaicin in Petterspray – Mode of Action"
A very in-depth background was presented on pepper spray. Pepper spray is available in almost every store, and in Germany it is available only for use against animals. (This brought up the question at the end as to whether pepper spray should be regulated as a drug rather than available "over the counter".) Strangely, pepper spray contains no pepper, rather the chili component, capsicum sp. He also pointed out that all the information to make pepper sprays (and more) is readily available on the internet. The major ingredients of the spray excites certain ion channels that can produce neuronal toxicity, but the events are poorly understood.

Conclusions
The overall message from this session was that both innocent control of civilians (such as pepper spray or the events that occurred at Tbilisi) as well as more threatening events (such as the Japan Tokyo incident that used impure sarin) require techniques to identify the toxic chemical agents used. Furthermore, a better understanding of the mechanism of action of the agents would provide a means to detect the agents better, and determine those agents that are indeed toxic. Most important, the standardization of the methodology to identify post-exposure to agents (biomedical samples) would provide the means to better enforce the Chemical Weapons Convention.

SESSION 10: CHOLINESTERASE DETERMINATION

Chair: Uri Wormser
Co-chair: Valerii Tonkopii

The estimation of activity of ChEs is a good mean for indication of pesticide intoxication in occupational health and therapeutic monitoring of various neurological diseases.
Determination of cholinesterase (ChE) is also a crucial factor in detection and monitoring exposure of humans to organophosphates and carbamates. Much effort has been invested in developing appropriate analysis that can simply and reliably determine ChE. The present session discussed the recent advances in this area. Dr. Eyer presented an improved method that, in addition of being easily performed, it gives reliable results down to 3% residual activity. Dr. Feaster demonstrated a new and accurate method for simultaneous determination of blood concentrations of both Acetyl and Butyryl ChE. This method was successfully employed in screening 320 blood samples in one four-hour time interval. Dr. Portmann discussed the reasons for the relatively high variations in Butyryl ChE levels obtained in different laboratories. He discussed several important technical improvements like the use of detergent and the loss of hydrocyanic acid in the reagent mixture. Dr. Simeon-Rudolf Described a simple method for Acetyl and Butyryl ChE using colorimetric method and DTNB for thiol groups determination. Dr. Brodbeck compared Acetyl and butyryl ChE levels in blood taken from capillary (finger) and from venous blood. He also tested the effect of different detergent on these enzymatic activities.

Thus, the session was important methodological step towards a common accepted methods, which may be applied in practice.

SESSION 11: DECONTAMINATION

Chair : Dr. Devendra K. Jaiswal (India)
Co-chair : Professor Slavomir Rump (Poland)

Decontamination plays a vital role in defence against toxic chemicals including chemical warfare agents. It is a process of conversion of the toxic chemicals into non-toxic or harmless substances by physical, chemical or biochemical methods, depending on the nature and type of contamination and surface on which it is dispersed.

The research & development in NBC defence obviously includes decontamination as an important area of work. The session had six research papers from distinguished scientists and technologists giving a wide spectrum of information on new developments on materials, methods and technologies for decontamination.
The decontamination of mass casualties after exposure to harmful liquid chemicals can be quite important in several situations particularly in an industrial accident. The casualties with traumatic injuries and exposure to toxic chemicals will create problems with regard to transportation, decontamination and medical treatment. Dr. Persson from FAO, Sweden in his paper on "Decontamination of casualties after exposure to harmful liquid chemicals" highlighted these aspects.
The Polyurethane foam linked mammalian cholinesterase for decontamination and detection of OP Nerve agents was an interesting development reported by Dr. Gordon et al from Walter Reed Army Institute of Research, U.S. Army Medical Research Institute of Chemical Defence, Aberdeen proving Ground and Centre for Biotechnology and Bioengeering, USA. It involves a bioscavenger approach to the protection against OP toxicity. A sponge product composed of ChEs, oximes and polyurethane developed for removal and decontamination of OP compounds from medically important surfaces such as skin and wounds. It can also act as a biosensor for detection of OPs.
Dr. Raton from Ervi Aerospace Canada Ltd. discussed large scale mass decontamination and special event planning criteria for special international events based on Canadian Aqueous System for Chemical, Biological and Radiological Decontamination (CASCAD) system and Canadian model. The CASCAD and its integrated family of COLPRO, Blast Guard and RSDL have been integrated into the response system to provide the latest decon technology available to counter any threat. The performance data for the system is based on extensive testing by DRES, DPG and TNO.

Recent and Planned Research to extend and further validate RSDL as a broad spectrum personal decontamination system for BCW agent decontamination had been studied by Dr. Philip O'Dell from O'Dell Engineering Ltd. Canada. The Reactive Skin Decontamination Lotion (RSDL) has been fielded in a number of countries for C agent protection of personnel. It is also useful for BW agent decontamination and for riot agent removal. Recent and Planned Research to extend further to validate RSDL as a broad spectrum personal decontamination system for BCW agent decontamination was described with the reports on the recent and planned testing studies in these areas.

Several decontamination systems have been incorporated in a Czech-made decontamination vehicle ST mounted on the undercarriage TATRA 815 for effective decontamination of heavy equipment based on either hot gases or wet processes using various solutions or emulsions. This development comes from Institute of Environmental Chemistry and Technology, Czech Republic. However, this paper was not presented as the authors Dr. J. Matouska and co workers could not make it to attend the conference.

The Acton International Ltd in conjunction with Defence Research Establishment, Suffield, Canada have made an useful development of new Chemical and Biological (CB) moulded gloves with far better tear resistance and physical properties and have very appropriately entitled that research paper as "Gloves that fit and do not tear. A novel idea". Major E. Lauri in his presentation highlighted the general composition of the material used in such gloves and highlighted its advantages of the new product.

SESSION 12: B-AGENT PROTECTION AND TREATMENT

The session was devoted to discussion of B-agent Protection and Treatment. Only small part of this huge field may be discussed in any meeting, and the reports presented in this session were related to several events.

Dr. D. Moore presented new promising biocidal resin Triosyn, characterizing by high potential of microorganisms destruction and simplicity, and convenience in practical application. The resin represents new approach to usage of iodine abilities to kill various microorganisms.

Dr. E. Ryabchikova presented current situation of smallpox researches, and proposed plan of variola virus studies in SRC VB “Vector” (Russia).

Prof. L. Haghighi’s topic was related to analysis of the infections among Iranian troops during Iran-Iraq war. Isolated agents were identified and their biological properties were examined. Resistance of the selected microorganisms to antibiotics was evaluated.

Dr. P. Russel’s presentation gave an overview of plague vaccine history, current situation and main directions for future. Plague is still dangerous disease requiring attention of epidemiologists and development of new modern vaccines. Modern approaches were discussed in the report.

The session was completed by Dr. J. Melling, who presented common principles of biodefense vaccinology. Biodefense vaccines are a special kind of vaccines, however, they should correspond to requirements for vaccines destined to public health. The report summarized general problems of biodefense vaccinology.

Taken together, the session paid attention to the most significant problems of B-agent protection and treatment.

SESSION 13, POSTER 2.

Poster Session 13, Chemical Poisoning, Treatment, Warning, Decontamination, was chaired by Keith Vesely and co-chaired by Michael McMillan and consisted of eight diverse poster presentations. Topics included: antidote delivery systems; effects of low-level inhaled sarin; development of adequate warning systems and risk assessment procedures in and around chemical demilitarization sites; present and future treatments for chemical warfare agents; and examination of mycotoxin levels in feed and animal livers.

Rene Pita explained the functioning, manufacturing process and technical characteristics of Spanish Armed Forces autoinjectors. New directions will include: replacing 1 ml syringes with 2 ml syringes; introducing HI-6 autoinjectors (research and development of two-chambered autoinjectors); research and development of combination atropine, oxime and anticonvulsant autoinjectors; and possibility of formulating autoinjectors with other drugs.

Jiri Bajger reported on a study examining the effects of sublethal concentrations of inhaled sarin on acetylcholinesterase activity in rats. Two relationships (i.e., sarin concentration vs. acetylcholinesterase activity and duration of exposure vs. acetylcholinesterase activity) were found to be linear. Results suggested that at low concentrations, acetylcholinesterase in blood is "titrated" by sarin. Acetylcholinesterase in the blood appears to be is a very sensitive marker for diagnosis of inhalation exposure to low concentrations of sarin.

Jiri Kassa described the long-term effects of low-level sarin inhalation in rats. Non-convulsive symptomatic levels of sarin can cause subtle long-term signs of neurotoxicity and immunotoxicity. When given repeatedly, clinically asymptomatic concentrations of sarin are also able to cause subtle, long-term signs of neurotoxicity. These sarin-induced long-term signs of neurotoxicity may be caused by mechanism(s) separate from the cholinergic nervous system.

Vadim Petrov described the need to replace obsolete warning systems to properly warn personnel at chemical warfare storage facilities as well as surrounding populations. Successful functioning of the plant for chemical warfare agent destruction in Russia will require the creation of updated security systems with the use of modern poison substances analysis methods, modern computer technologies and means of communication.

Manana Juruli evaluated the health risk of the "old military west" in Georgia. To prevent human health against harmful impact of hazardous uncontrolled chemical waste, risk assessments of located contaminated sites must be provided and the national legal instruments related to chemical management must be improved.

Ashima Saxena presented work establishing a relationship between ligand-induced acceleration of acetylcholinesterase reactivation and phosphoryloxime inhibition of the reactivated enzyme. Results suggest that cholinesterases, oximes and organophosphorus hydrolases can work in tandem to hydrolyze or inactivate organophosphorus compounds in vitro and in vivo.

Heyballah Kalantari presented a study designed to determine the extent of contamination of mixed feed by mycotoxin and the subsequent presence of the toxin in livers of poultry fed the contaminated feed. Results of the study showed alfatoxin contamination was present but that the levels were safe. Production of mycotoxins may be accelerated by improper production and handling of feeds.

Gurayten Ozyurt described the role of serum cholinesterase and the Namba classification in diagnosing and managing organophosphorus intoxication in humans. Clinical appearance was found to be more important than serum cholinesterase levels in determining when to cease antidotal therapy, because it will take at least 4-6 weeks the enzyme to return to normal levels. In fact, some patients may have low serum cholinesterase activity without any symptoms.

SESSION 14: POSTER SESSION 3: BIOLOGICAL AGENTS AND TERRORISM

Chair: Lisa Rotz
Co-Chair: Maarten Nieuwenhuizen
Poster Presenters: U. Huber, V. Yousefi, N. Schürch, R. Gordon, Dj. Jovanovic, and S. Filer

In this session, the authors presenting posters in session 3B began with a brief oral presentation of their posters before moving into the poster viewing room to answer questions. The posters in this session addressed a variety of subjects from policy approaches to combating chemical terrorism to specific methods for detection and early warning for chemical and biological agents. In addition, research projects that evaluated countermeasures for botulinum toxin B and T-2 mycotoxin were presented.

Huber described the work of a working group assigned by the Switzerland national board for NC protection determined the low probability for chemical terrorism did not justify maintaining their population under continuous alert. Their recommendations were to maintain the required regulations and safeguards that are in place for general industrial chemical accidents and assure that the responsible industrial officials maintain their own response capabilities. In addition, a national team of 50 experts from the AC-Laboratorium in Spiez would be available at all times for rapid deployment and consultation to these industrial response teams if needed. In addition, stores of atropine for the treatment of nerve agents would be held among others at the AC-Laboratorium for rapid deployment if required. Discussion regarding the rapidity of response took place. The expert team would be expected to be onsite of any chemical disaster in Switzerland if requested within 2 hours. The observation was made that the problems and approaches for responding to chemical terrorism seem to be similar for various countries. Discussion occurred regarding the necessity of each country developing their own solutions, or could a common response template be developed that may then be used by several countries.

Research regarding the development of a detection method for biological agents and toxins using monoclonal antibodies was discussed by Schürch et al.. The authors of this poster `immunized rabbits using an attenuated strain of *F. tularensis* or used a semisynthetic method to develop antibody libraries. The semisynthetic libraries used mRNA converted to DNA then random recombination of heavy and light chains to develop a large library of antibody DNA. These were then introduced into plasmids and packaged by bacteriophage which were then used to introduce the DNA into *E. coli* for expression. *F. tularensis* specific antibodies were then selected using an ELISA antigen capture method. The antibody libraries created using the animal model and the semisynthetic method were compared and appeared to be similar in their specificity for *F. tularensis* antigens. The authors determined that it is possible to develop specific monoclonal antibodies for the detection of biological agents and toxins using non-immunogenic molecules and random recombination libraries. The author discussed the difficulty in preventing the denaturation of the antigen for the capture method and feels that using the ELISA capture method has prevented denaturation. Further testing will confirm this.

Buforin I (a peptide from the stomach of Asian frog) was found to be a competitive inhibitor of botulinum toxin B by Gordon et al. who evaluated several peptides that contained the QF cleavage site. Botulinum B toxin was shown by the authors to cleave synaptobrevin (VAMP2) at this site and peptides that contained a similar site were studied for their substrate or inhibitory possibilities. Buforin I functioned as a competitive, dose-dependent inhibitor for botulinum B toxin activity but not as a substrate. Several subset peptides of Buforin I were also evaluated and found to have less competitive activity than Buforin I. Sequence analysis revealed an 18% conserved amino acid similarity between botulinum B toxin and Buforin I while computer assisted secondary structure predictions showed two stretches of similar helical structures flanking the cleavage site of both substances. Discussion regarding the delivery of the peptide for effectiveness occurred. The authors are currently evaluating possibilities which include connecting the peptide to a chain which normally delivers it into the cell or separating the heavy and light chain from the toxin before it gets into the cell. In addition, the inhibitory effects of Buforin I to other botulinum toxins has not been evaluated at this time but further studies are planned.

A method for assessing the risk of different agrochemicals for use as a terrorist weapon utilizing an analysis of their availability, volatility, and human and animal toxicity was presented by Yousefi. Ratings of high, medium, or low for each category were assigned to the agrochemicals currently available for industry use in South Africa and this scoring system was used to determine which chemicals present the greatest risk. Comments were very positive regarding the rating scheme used and questions revolved around the amount of interest other countries have expressed in adopting this method.

The efficacy of multiple treatment regimens utilizing corticosteroids in rats exposed to T-2 mycotoxin was evaluated by Jovanovic et al. The protective index of a regimen was the ratio of the LD50 values in treated and untreated animals. The greatest protective index (3.37) was seen with regimens that utilized corticosteroids administered in

equal doses at 24, 18, and 1 hour before, and 6 hours after exposure to the mycotoxin. However, a protective index of 2.5 was also seen with corticosteroids only administered immediately after the exposure to the toxin. The dose of corticosteroid but not the route of administration produced significant differences in the protective index or the regimen.
A novel strategy using synthetic nucleic acids combine with conventional immunoassays for biological agent detection was described by Filer and Steven. The subject matter presented was a hypothetical strategy to respond to significant gaps in current technology with regard to timely biological detection. The stated technology could be viewed as proactive, rather than reactive, in concept and design. The poster presentation was a description of a Multiplexed Detection System that would serve as a bridge between two distinct assay technologies in an attempt to ameliorate the limits endemic to current immuno-diagnostic systems. The assay employs the use of synthetic DNA and non-toxic proxies for the target organisms. With the capability to perform up to several hundred concurrent assays on a single sample, it would limit both false positives and false negatives. The strategy described in this poster presentation was conceptual and not a solid-state technology at the time of presentation. Discussion regarding whether this system would work for genetically altered organisms occurred. The author felt that it should still work in theory as the detection mechanisms are aimed at virulent characteristics of the organisms that would most likely need to be maintained in order for the organism to retain its virulence.

SESSION 15. BIOSCAVENGER

In this session on Bioscavengers, six speakers presented research on the development of stoichiometric bioscavengers such as cholinesterases (ChEs) and catalytic bioscavengers such as OPAA and OPH.

Dr. Doctor demonstrated the use of three different types of cholinesterases such as HuS BChE, Eq BChE, and FBS AChE in protection against OP intoxication in four animal species (rats, mice, guinea pigs, and rhesus monkeys).

He was followed by Dr. Broomfield, who described the kinetics of nerve agent hydrolysis by human OPAA hydrolase and demonstrated that despite the high Km, this enzyme can catalyze the rapid hydrolysis of soman and sarin, and to a lesser extent VX.

Dr. Cheng described his studies on the isolation of an OPAA hydrolase from *Alteromonas*, its characterization as a prolidase, and the cloning and large scale production of recombinant enzyme. He also described the formulation of recombinant enzyme in different matrices for possible use in chemical agent decontamination, personnel protection. and detoxification.

Dr. Saxena discussed her attempt at improving the bioscavenging property of Mo AChE by site directed mutagenesis studies and demonstrated that the E202Q AChE was 2- to 3-fold more efficient than wild-type AChE in detoxifying soman and sarin compared to wild-type AChE. However, the recombinant enzymes had a much shorter mean residence time in the circulation of mice as compared to plasma-derived ChEs, which could be correlated with the structure of glycan moieties on these enzymes.

Dr. Way demonstrated the use of recombinant OPH encapsulated into sterically stabilized liposomes (SL) in the presence of 2-PAM and atropine, for the protection of mice against a 1000 LD50 of paraoxon.

In similar studies, Dr. Petrikovics demonstrated the use of recombinant OPAA encapsulated into sterically stabilized liposomes (SL) in the presence of 2-PAM and atropine, for the protection of mice against DFP.

SESSION 16 – TERROR

Chair Ladislaus Szinicz, Co-chair Andrew Robertson

Various presentations from other sessions (No. 3 – Bioterrorism; No. 7 – B-Threat and Detection; and No. 14 – B-Agents and Terrorism) have already stressed the disastrous results that may arise from terrorist or non-state use of chemical and biological warfare (CBW) agents.

Mitigating the impact of a CBW attack requires timely planning, properly trained personnel, rapid detection methods, protection and medical equipment, pharmaceuticals, dedicated facilities and a rapid, and well trained. emergency response organization. Any reaction to a CBW terrorist attack will be infused with high levels of anxiety.

emotion and psychological pressure. Dr. Gui Santana (Abstract 2) noted that intensive communication at different levels is inevitable in any incident response and stressed the importance of the early "hardening" of any disaster communication system.

Intensive education and training, coupled with appropriate equipment, is also important. Col. Centonze (Abstract 57) outlined the particular CBW protection and detection training which was provided by the Swiss Government and emphasized the benefits of cooperation between civil and military authorities

Dr Lisa Rotz (Abstract 89) addressed the evaluation and prioritization of potential biological agents against objective criteria to ensure appropriate allocation of the limited funding by public health authorities. By identifying priority agents, public health programs can be targeted appropriately.

.Dr. Lindsey (Abstract 83) stressed the importance of the availability of standardized laboratory protocols during a CBW attack and outlined the approach CDC had taken to make this information available using the internet. Dr. Threadwell (Abstract 92), using the Seattle World Trade Organization meeting as an example, emphasized the need for surveillance programs for early recognition of characteristic symptom clusters, which may be the first indication of terrorist use of biological agents.

The daunting task of improving protection against CBW terrorist attack will be more cost-effective with continued national and international cooperation.

1. BIOTERRORISM AND AUSTRALIA - THE WAY AHEAD

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INTRODUCTION

Bioterrorism, the deliberate use of biological weapons by a terrorist group, has become a topic of interest for Australian medical, government and military agencies over the past two to three years. The various forms of media, from reputable newspapers and journals to novels, documentaries and films, regularly depict such scenarios without attempting to develop a realistic and balanced appreciation of the threat. In Australia, this depiction has varied from fairly balanced national newspaper articles (1) to Tom Clancy's more outlandish 'Rainbow 6' portrayal of terrorism at the Sydney Olympic Games. (2) Whilst response planning to a bioterrorist attack has been a major issue in the United States since 1996 (3), Australia is now coming to terms with the issues involved and the possibility of Australians being a target.

The worldwide threat of bioterrorism is increasing and, despite its geographic isolation and limited history of terrorist attack, Australia can not expect to be excluded from this trend. The dimensions of this threat need further exploration to enable a realistic appreciation of the response required. (4) Such a review will enable an objective appraisal of Australia's current response capability and what future capability is needed.

BIOTERRORISM

Biological weapons have been utilized in one form or another for over 2000 years. (5) Despite advances in detection and therapy, biological warfare remains a threat on the modern battlefield. The Russian and Iraqi biological warfare programs have shown both the utility of and the ease with which covert programs can be hidden. (6) Biological weapons may also prove to be a useful weapon in the armamentarium of the terrorist groups. The Aum sect has both researched and, unsuccessfully, tried to use anthrax and botulinum toxin. (7) Other terrorist groups, like Usama bin Laden's organization, have indicated a strong interest in acquiring these agents as weapons. (8)

So is it just a matter of time before Australia faces 'Bioterror'? (9,10) Various press articles and novels would certainly have us believe that. To gain an appreciation, however, of whether these claims are realistic or merely sensational, a review of the current trends in terrorism and biological warfare is useful. The media hype and claims, that biological weapons are so easy to produce and use that they have become the veritable terrorist 'poor man's atomic bomb', (11,12) are not harmless and may even produce some untoward effects. One unintended effect of the sensational media depiction of these weapons has been to make them more attractive to hoaxers, as demonstrated by the continuing spate of anthrax hoaxes in the United States. (13)

THE TERRORIST THREAT

Terrorism, 'politically or religiously motivated acts of violence causing non-combatant casualties', (14) has largely spared Australia. (15) Australia has seen small scale terrorist attacks, like the bombing of the Sydney Hilton in 1978, and various extortion attempts and hoaxes, including the 1984 threat to release foot and mouth disease in Queensland. Compared to many countries, however, Australia has been relatively immune. (11,15)

The terrorist threat is changing. In the 1970s and 1980s, terrorist groups, whilst radical politically, were far more interested in getting their message across than killing lots of people. (16) The nineties saw a fundamental change. While the number of terrorist incidents has continued to fall, there has been a paradoxical rise in the percentage of incidents with fatalities. This trend is associated with the growth of radical religious terrorist groups. These groups, arising from a wide spectrum of religious backgrounds, see violence as a `divine' duty and are not constrained by the political or even `moral' constraints of the more traditional terrorist groups. (16,17) Able to contemplate far more deadly attacks to fulfil their aims, these groups have come the closest to effectively using weapons of mass destruction. The deliberate 1984 infection of an Oregon town's population with Salmonella typhimurium, by the Bhaghwan Shree Rajneesh cult, was a forerunner to the Aum Shinrikyo sect's more deadly 1995 nerve gas attack on the Tokyo subway. (18) The more traditional terrorist groups, however, should not be ignored. Non-attributable bioterrorist attacks against animals and plants may achieve their aims without accruing the same retaliation. (10) Although there has been no major terrorist attack with biological weapons, many believe that this situation will not last. (11,16,18,19)

BIOLOGICAL EXPERTISE

Although opinions vary, most authors believe that an individual or individuals with a modicum of technical skill could acquire the necessary expertise to produce biological weapons in small quantities. (11) Whilst terrorist groups in the past may not have had the necessary technical expertise, (16) the previously esoteric skills required are now generally available. American industry employs around 60,000 biologists and there are nearly 1,900 biotechnology companies in the United States and Europe. (20) Similar expertise exists within Australia

As we have seen from the past Russian and Iraqi biological warfare programs, interest and expertise exists within a number of proliferant countries. (6)

THE THREAT TO AUSTRALIA

Australia, on the surface, does not appear to be a probable target for a terrorist attack, let alone a bioterrorist attack. Our close association with the United States, an avowed Usama Bin Laden target, (22) the general openness of our society and the spectacle of the 2000 Sydney Olympics, with a host of possible targets, may make Australia more attractive for terrorists. Whether such terrorists will resort to biological weapons is a more vexing question. There are good reasons for terrorists not to use biological weapons. The inherent unpredictability of biological weapons, a personal fear of biological agents, anticipated governmental response to an attack, and a general satisfaction with current measures, may contribute to the terrorist's reticence. (11,16) The fear of collateral damage to friends and a desire to limit the number killed may also concern more traditional terrorist groups. (11) Most authors agree, however, that with the growth of religious terrorism and availability of agents, there will be future attempts, on a limited scale, to use these weapons. (11,16)

Given intent, the next consideration is capability. A terrorist group, even with limited technical capabilities, may be able to acquire or import a biological agent into Australia. Potential biological warfare agents could be acquired from natural reservoirs, appropriated or stolen from medical or research facilities, bought from legitimate or 'black market' suppliers, or procured from 'unfriendly' governments. (11,22) Recently, concern has been raised about the potential use smallpox as a terrorist weapon, however, limited availability would make it difficult to acquire and use. (19) Interest has already been shown by various overseas terrorist groups in acquiring anthrax, botulinum toxin and ricin. (17,23)

Whilst terrorists with a modicum of technical training could produce biological weapons in small quantities, (11) the necessary technical skills and equipment needed to produce biological agent in large quantities, to weaponise that agent and to utilise the agent effectively, are far more difficult to procure. (4,13)

Delivery is the most critical step. Most authors agree that effective delivery of biological warfare agents is even more problematic than its production. (20) The most commonly proposed means of terrorist delivery is by the spread of an aerosolized suspension of biological agent over a city. (7,14) This process, fortunately, is more difficult than it first appears. (12,24) There are significant technical problems in keeping a biological agent viable in a cloud for long enough to infect or intoxicate the victim. (12) Humidity, sunlight, smog, temperature and winds will all affect the final dose received. (25) Even with a good technical background, the Aum sect was unable to produce any biological casualties despite several attempts to deliver anthrax and botulinum toxin in Tokyo. (24,26) Whilst a smaller scale attack in a confined area may be more feasible, the technical ability to produce and weaponise sufficient agent to cause harm would probably be beyond the capabilities of most terrorist groups. (11)

The poisoning of a large water reservoir is also not as simple as postulated. An attack would require large quantities of agent and is unlikely to be successful due to problems with access to the site, dilution and environmental degradation. (11,27)

THE RESULTS

A successful bioterrorist attack has the potential to be disastrous. Even a relatively small attack might quickly overwhelm the resources of even the richest and most capable of countries. (28) In an attack, death and disease would be only part of the problem. The potential psychological effects and resultant panic would adversely affect the infrastructure and operations of any country. (9) The financial impact would also be monumental. Kaufmann's model of the economic impact shows that, for every 100,000 persons exposed in an anthrax attack, the financial cost to a country could be over 26 billion American dollars. (29)

AUSTRALIAN DEFENSE AGAINST BIOLOGICAL TERRORISM

Australia faces the challenge of many developed nations. The face of terrorism is changing and Australia, like most countries, is unlikely to be immune in future. The move to religious terrorism increases the probability that future terrorist attacks will involve biological or chemical weapons. Improved technical skills and equipment make a bioterrorist attack both more probable and more likely to be successful, particularly if done on a small scale.

Defending against biological terrorism is a daunting task. Unlike chemical or nuclear weapons, the current biodetection systems are limited in their scope and availability, so emphasis has to be placed on other measures. Sensitivity analysis has shown that preventive programs are cost-effective in defending against biological terrorism. (29)

The Australian community has, until recently, been generally under-prepared. In the early 1990s, there was no policy, training or planning in this area. In 1998, the Australian Medical Disaster Coordination Group identified major deficiencies in the preparations for a chemical or biological terrorist attack and set out, with

Emergency Management Australia, to rectify these deficiencies. (30) Australia is now preparing for biological terrorism, and its defensive measures can be broadly grouped into four main areas. (11)

The first area is information collection. Security and police agencies monitor, and will continue to monitor, individuals and terrorist groups of concern. Sydney police recently arrested a man who threatened to shoot down planes during the Olympics. (31) Australia is also fortunate in having a very well developed Public Health laboratory network, which both catalogues local endemic and epidemic disease and rapidly identifies and responds to epidemics through its surveillance network. (32, 33) This system provides input into the ProMED internet epidemic surveillance system, which has been very effective in establishing baseline data. There are well-developed links between different Government agencies and the medical community, (32) through Emergency Management Australia and committees like the Australian Medical Disaster Coordination Group, which ensure that information is shared to assist in creating a coherent picture of the problem. (33)

The second area involves counter-acquisition strategies. Countries must make it very difficult for terrorist groups to acquire biological weapons. Australia has been at the forefront of such strategies over the last decade. Australia established the Australia Group, a group of like-minded nations, who monitor and control their national export of chemicals, biological agents, precursors and dual-use equipment. Australia has also strongly supported the establishment of the Chemical Weapons Convention, the verification protocol for the Biological Weapons Convention, and the United Nation Special Commission, who were responsible for disassembling Iraq's biological weapons program. While each of these strategies is not an end in itself, they contribute to the counter-acquisition web, making the acquisition and use of biological weapons more difficult. Counter-acquisition may require countries to both threaten, and be willing to carry out, retaliation against the terrorists and their state sponsors should biological weapons be developed or used. (34) Through participation in sanctions, Australia has been involved in bringing economic and moral pressure to bear on countries like Iraq to comply with international conventions, including the Biological Weapons Convention. (6) The availability and movement of dangerous biological agents and microbiological equipment is regulated and tightly controlled by Australian Customs and the Australian Quarantine Inspection Service and trade in this area is heavily regulated. Australian Police are receiving the necessary training to identify these agents, and the equipment needed to produce and disseminate them, so they may be apprehended in the acquisition and transport stages. Australia also has a well-developed and nationally agreed National Anti-terrorist Plan. This plan is regularly exercised and revised. (35)

Passive protection is the third area. New, innovative and rapid biological detection systems are a cornerstone of early and appropriate response. (19) The Australian Defense Force (ADF) has made substantial progress in this area following the Gulf War. The Defense Science and Technology Organization (DSTO) commenced research into medical defenses against biological weapons in 1995 and have made some excellent advances in the development of bio-detection systems. One of the most promising is the AMBRI biosensor, which can rapidly detect up to four agents at a time.

Similarly, effective disease surveillance systems are critical. Adequate epidemiology and pathology resources are key facets of this surveillance. (28) The current Australian noticeable disease system is very effective and able to detect acute changes.

Protection should also cover the stockpiling of vaccines and therapeutic agents; improved water supply, air-conditioning and food production security; development of better individual protection equipment, and increased research into medical defenses against biological weapons. (11, 23) DSTO has also contributed to the development of other detection systems, therapies for the management of biological attack and improved individual protection equipment. The new lightweight suits, whilst giving the protection of older suits, will markedly reduce the heat stress encountered in the Australian environment.

In November 1998, NSW Health identified a project manager to coordinate its response to chemical and biological threats. A review of equipment and standard operating procedures and an audit of drug supplies have been initiated. Various other States are also looking at protective equipment and detection requirements.

Finally, there must be measures that mitigate the effect of an attack. These measures, including better and more specific biological disaster planning, public health coordination, and evacuation planning, are all being developed in Australia. (11) Emergency and medical responders are learning about what they may be dealing with and how to manage it. (23,34)

The ADF is able to decontaminate and render safe chemical and biological munitions whilst protecting its forces through detection systems, protective equipment, medical countermeasures and research. Doctrine for the management of biological munitions and casualties has been developed, and instructors, specialist advisers and medical officers trained. Such preparations, however, are focused on troops in the field and not on terrorist threats. The Commonwealth Government committed $23 million in the last budget to enhance this capability, with increased spending on response capability and protection and detection systems.

The Commonwealth Government is regularly exercising chemical and biological terrorism disaster plans through desktop and other exercises. (36) Commonwealth Health has completed an Australian Emergency Manual, which provides doctrine for the management of chemical, biological and radiological casualties. This

manual, coordinated by Emergency Management Australia (EMA), is in the process of being released. (37) EMA has also provided awareness training material to all the States and Territories. (37) Further health service training commenced in late September 1999 and has continued in a variety of forms throughout 2000. This training was developed utilizing the manual and assistance from States and Territories through the Australian Medical Disaster Coordination Group. All States and Territories are looking at the contingency planning, health co-ordination and training requirements of such preparedness, with a number of States training emergency response personnel throughout 2000.

CONCLUSION

Bioterrorism will not disappear as a potential problem and will remain an area of political and media interest. Fortunately, the successful completion of even a small-scale bioterrorism attack is far more difficult than portrayed by much of the media. Even a very limited attack, however, may have a major psychological effect with the resultant panic severely hampering any emergency response. The Australian medical and emergency response communities are facing this threat and will be better prepared to manage both the bioterrorist and emerging infectious disease challenges of the new century.

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KEYWORDS

bioterrorism; terrorism; Australia; defense; analysis

2. COMMUNICATION IN THE HEAT OF A CRISIS - THE CRUCIAL ELEMENT FOR EFFECTIVE CRISIS DECISION MAKING

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ABSTRACT
Chemical or biological accidents or threats (terrorism, etc.), given their devastating destructive potential to both mankind and the environment, are always a matter that requires immediate attention and response from a number of different stakeholders. It inevitably involves a great deal of communication and coordination between those stakeholders directly involved in attempting to neutralize a threat or the effects of CB accidents. Those situations are invariably characterized by high level of anxiety and emotional pressure which may compromise quality decision making. This paper looks at some effects of crises on organizations and how they, in turn, affect the critical issue of communication during a crisis. The paper also proposes a crisis communication model.

INTRODUCTION
A close observation of the crises events in the last few years suggests that not only the number but also the scale and magnitude of industrial and commercial crises are on the increase. The devastation caused by recent crises included the loss of thousands of human lives as well as immeasurable damage to the environment and future generations. In fact, man-made crisis today have the potential to rival major natural disasters. Crises also respect few if any boundaries, as several recent crisis events illustrate.

While some authors suggest that the dynamics of modern life have created a risk society (1) and that the potential for large scale human-induced crises are virtually built into the fabrics of our times (2), others contend that much of our current fear is groundless and not based in objective reality, but rather stems from the modern condition (3).

Regardless of the perspective adopted to explain causal issues, it is clear that no organization is immune to crises (4) (5). Moreover, the fact that the potential negative effect of technology exceeds the ability of our organizations and management structures to control them provides an enormous challenge to the central core of management. Organizations, therefore, should provide answers to three essential questions: (a) what role management plays in the incubation of crisis potential?; (b) what role management plays in the prevention and management of crisis events?; (c) why do many organizations and managers still deal with the issue of crisis in a superficial manner? Inherent to all these questions is the critical issue of organizational communication. This paper argues that organizational communication is the critical element in both crisis prevention and management. In essence, managing a crisis is managing communication. There are, however, organizational issues that inhibits effective communications (6) (7) (8). If these blocking elements in the system are not removed or eradicated, so as to guarantee information quality and flow, the effective management of crisis becomes an almost impossible task.

CRISIS, DECISION-MAKING AND ORGANIZATIONAL COMMUNICATION
Crises occur as a consequence of the dysfunctional nature of organization's culture, core beliefs, values and basic assumptions of decision markers and the approach taken to both external and internal communication. A crisis evolves through a pattern of different phases, each with its own distinct features and dynamics, that demands specific management approach (9) (10). Since the status of a crisis (intensity, scope, effects on stakeholders, etc.) changes as it develops within each phase, the various stakeholders involved in managing the crisis need to be constantly informed of crisis status so that they could act (decision making) in a concerted and effective way. Communications, then, play one of the most important roles in the process of administering and resolving crises.

Decision making is an integral part of crisis management process (11). The quality of decision depends greatly upon the quality of information available to the decision process which in turn depends on how and when (timing) it is communicated to the decision making person or team. That is, if communication is not effective and timely, quality information is of no use or value. Communication is regarded as the „essence" of crisis management. Without a well conceived and implemented communication system chaos reign. There are, however, many factors that may constrain communications in times of crises.

A typical feature of crisis is information overload. The quality of information input into the decision process depends on the ability of the system to effectively absorb information flow, thus preventing overloads. Information overload results in dysfunctional selective attention, retention of information, and delays and subversion of

communication flows. Information overload and the need to act quickly cause decision makers to use fewer communication channels for the collection and dissemination of information. Limiting the search for information can be disastrous. Divergent searching increases the variety and quantity of alternative solutions, and is essential in poorly structured circumstances in which fluency and flexibility of thought are vital.

When communication is not effective, the information content of the messages is frequently distorted because intermediate message-handling units omit, delay, filter, and most often process incorrect information. As a result, the decision group not only has fewer creative solutions available to it, but it is also more likely to fashion flawed solutions from the information they have.

CB crises invariably exert a great amount of emotional and psychological pressure on decision makers. See Figure 1 showing the consequences of crisis at psychological and emotional levels.

The very thought of a CB crisis may become enormously complex for those responsible for managing and coordinating the large amount of required effort. Variables, explanations, consequences, causes, relationships, alternatives, participants, goals, and potentials form a dense mass. During a CB crisis stress is of such magnitude that it promotes dysfunctional behavior. An increasingly severe crisis tends to make creative policy both more important and less likely. Decision makers find it harder to reason abstractly and are also less able to predict the consequences of various alternative courses of action. All this contributes to a restricted and distorted understanding of the decision situation. As a result, unaided crisis decision makers tend to make and implement inferior decisions.

See Figure 2. the consequences of crisis at an organizational level. In this scenario, open communications channels and quality information are the only hope for a positive outcome. Effective internal communication enables management to make sound decisions and be in control of the situation whilst effective external communications allows management to tell their history avoiding rumors and pseudo experts accounts. The net results are often a faster resolution of crisis, less internal anxiety and higher commitment to crisis management, less speculation and scrutiny from various external publics, among others.

Communications during crisis differ substantially from communications in „normal times". Those involved or responsible for dealing with CB threats and crisis need to have in place specially designed communication strategies and structure for managing adverse situations. It is virtually impossible to learn about crisis communications or to invent things in the heat of a crisis. As crisis evolves, each phase requires different management treatment and approaches. The amount of attention given to each particular issue is clearly an strategic decision of the organization and each decision taken will inevitably reflect on the status of crisis and on its resolution potential.

Effective crisis management starts well before an issues erupts into a full blown crisis. Indeed, it is much safer to manage the symptoms than the chain of complications resulted from a crisis. Communications, then, plays a vital role in crisis prevention. Most major crises to date (including some well-publicized CB crises) could have been prevented if the organizations involved had in place effective communication systems. Communication system in this sense means both formal and informal communications. As mentioned previously, organizations, depending on the nature of their culture and core beliefs, have a high potential for crisis incubation. Therefore, understanding and de-codifying the unwritten organizational rules and norms seems to be of crucial value for crisis prevention and management. If an organization has a crisis-prone culture, the incubation of crisis will occur as a function of the strategic decision making that takes place within the organization and the failure of management to identify and acknowledge the limitation of its control mechanisms. A crisis-prone culture inhibits communications that, in turn, severely weakens management's ability to develop resilience within the system. In another words, it deprives an organization from the essential abilities and mechanisms needed for developing preventive measures and strongly influence the psychological, emotional, and technical „abilities" of its member to manage the crises that occur.

Considering the evolutionary nature of a crisis, a communication model that contemplates all phases of a crisis has been develop. The model proposes that open lines of communications should be established to and from the environment. The effectiveness of crisis management depends not only on the efficiency of the formal and informal communication structures but also on the organization's ability and capacity to absorb, filter, process, manage, and implement strategies based on the information received. See Figure 3, for a simplified crisis model.

Crisis communication training is essential. CB crises invariably arouse a great deal of emotions and scrutiny from all sectors of society is unavoidable. Different stakeholders require a different set of information to satisfy their particular interests or concerns. The establishment of a crisis management communication center is imperative.

CONCLUSION

Considering that crisis is an unavoidable organizational phenomenon, and that organizational ability and capacity to avoid and managed crisis has been identified as limited, as evidenced by a close examination of the average life-span of companies, the characteristics of highly visible crisis episodes and the numerous examples of dysfunctional behavior of organizations, this paper has examined the role communications (formal and informal) play in times of

crisis, emphasizing its importance for effective decision making for crisis prevention and in averting further chaos.

Crisis management requires previous preparation. It is virtually impossible to develop or invent strategies in the heat of a crisis. Organizational culture (core beliefs, values and basic assumptions) dictates how an organization deals with the issue of crisis incubation and crisis emergence. Thus, organizational culture influences how communication is structured within a system as well as how decisions are taken. If communication is not effective, unaided decision-making may lead to further aggravation of a crisis.

Finally, given the negative potential effects of chemical and biological accidents, the public perception of CB operations, and the high degree of scrutiny and speculation that CB issues receive (in times of crisis and non-crisis), organizations in the sector are always in a delicate position. Therefore, crisis communication should be treated as a continuous effort. Indeed, effective communications start well before a crisis erupts.

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KEYWORDS

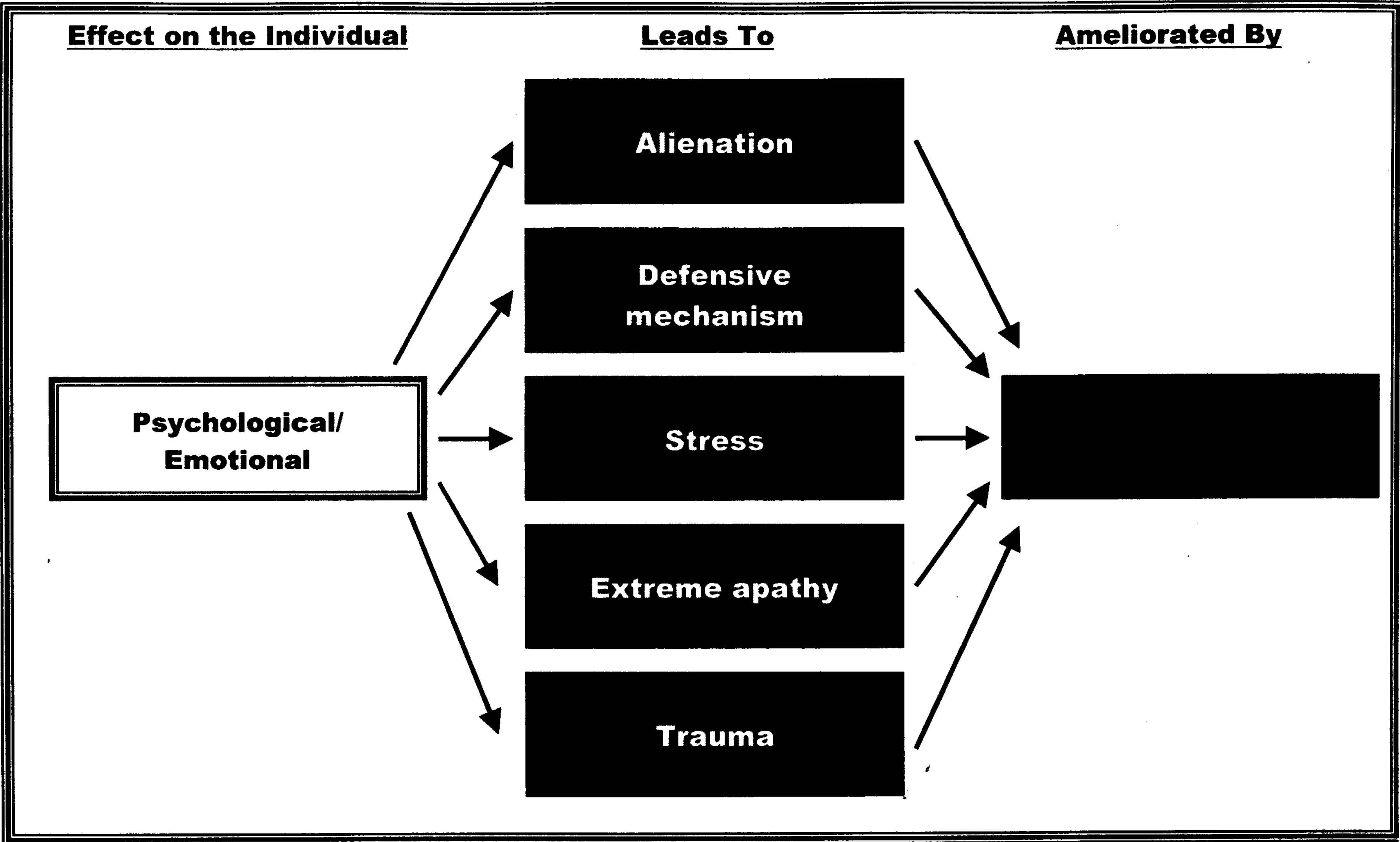
Crisis, crisis management, crisis communication, stakeholder, crisis communication model

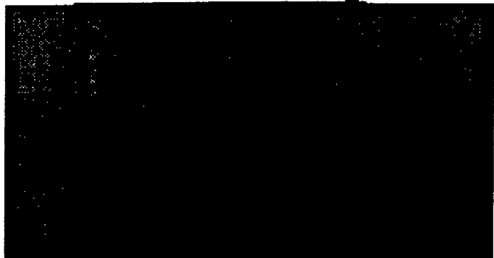
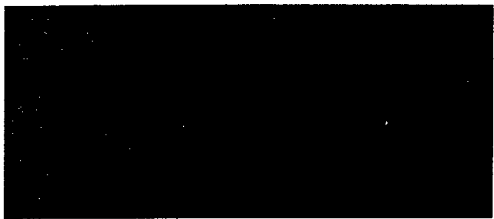
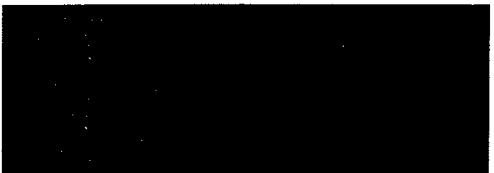
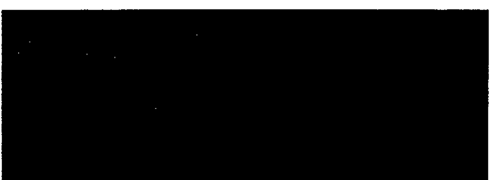
FIGURES AND TABLES

Figure 1 – The Consequences of Crisis at a Psychological and Emotional Levels

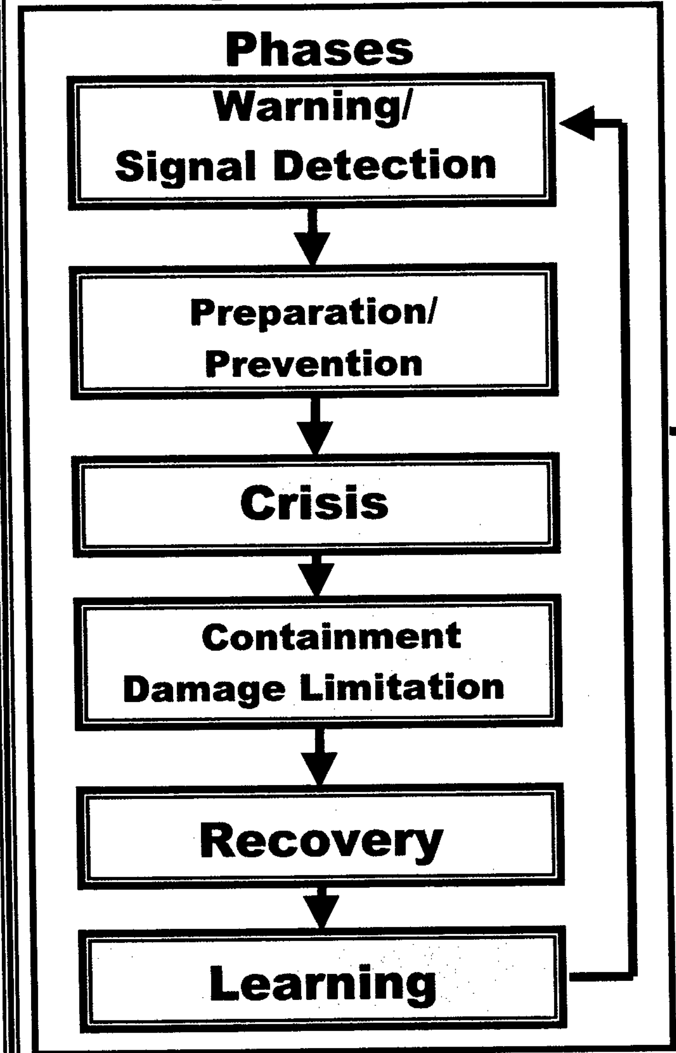
Figure 2 – The Consequences of Crisis at an Organizational Level

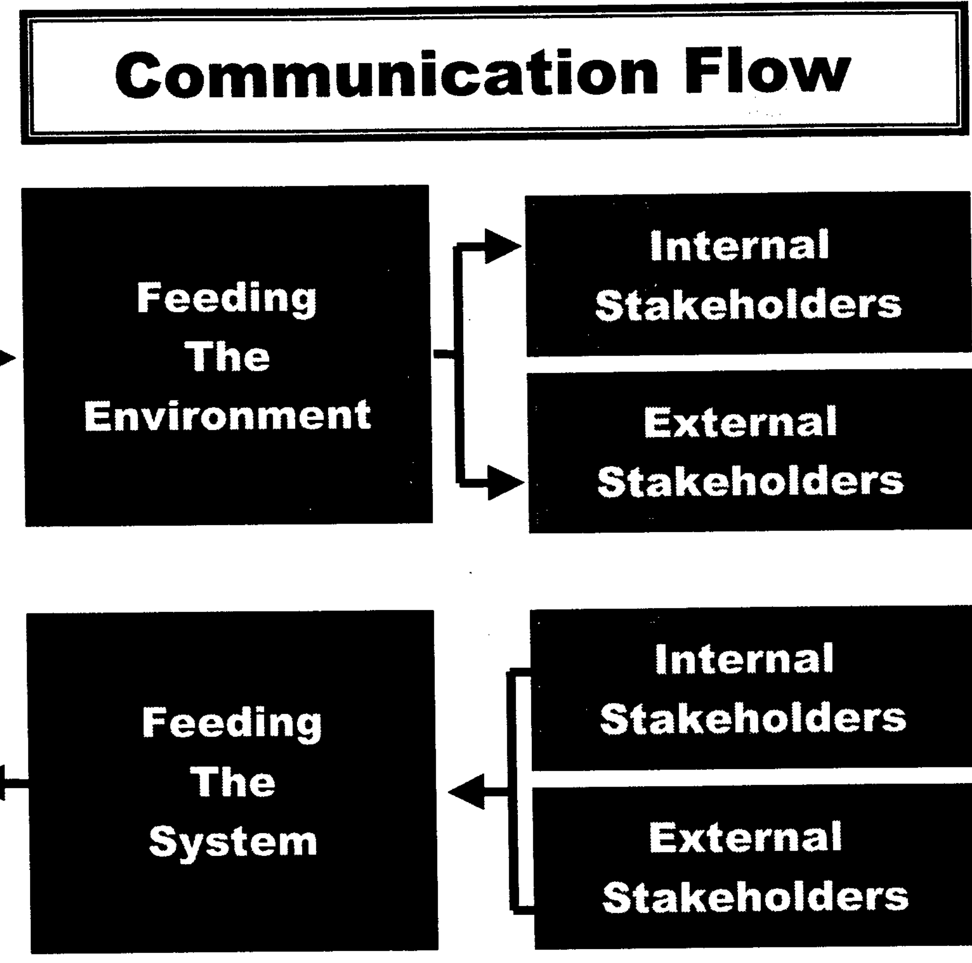
Figure 3 – A Simplified Crisis Communication Model


Effect on the Individual
Leads To
Ameliorated By
Psychological/ Emotional
Alienation
Defensive mechanism
Stress
Extreme apathy
Trauma

Effect on the Organization	Leads To	Ameliorated By
Centralization decision making authority	Group-think	
Constriction in information processing	Routine decisions Few decision alternatives Flawed decisions	
Regidity in response	Inflexible decision rules Inappropriate decision rules	
Conflict in resource management	Delay in decision implementation	
Pressure for explanation	Frustation Anxiety	

SIMPLIFIED CRISIS COMMUNICATION MODEL





PHARMACOLOGY AND TOXICOLOGY OF HI-6

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SUMMARY

In a number of countries apart from just the scientific research the Hegedorn oxime HI- 6 became used also practically. This use is connected with the preparation of drugs which are being tested according to the legislature of the different countries. The Bulgarian liophile ampoule of HI – 6 (1986) showed a lower toxicity compared to other oximes. The results in this regard are close to the results obtained by other authors in their work with HI – 6. The studies on the experimental animals show that i.m. application does not cause statistically significant differences in blood pressure, the frequency of the heart activity, breathing, and the EKG data, in comparison with the controls. After i.v. injection (bolus) a short period of deepening of the breathing and falling of the blood pressure is observed with about 40-50 mm Hg. After 5 min. the above mentioned parameters go back to their normal levels. In the EKG study during the same period a light bradycardia has been established. Deviations in the laboratory test during the study are not observed. Pathomorphological studies on the 24th ^{hr}, 15th and 30th day after application of a single dose HI – 6, do not show change in the investigated organs as well as on the places of the injection. Our pharmacokinetic research shows that HI – 6 is being eliminated quickly by the organism. In connection with that process and also with the changes of the pharmacokinetic's of the HI – 6 after intoxication with OPC, it is recommended that HI – 6 is being monitored in clinical conditions.

INTRODUCTION:

The H-Oximes, so called to the person, who synthesized them for the first time with her team, Prof. Ilze Hagedorn. Moreover, she and her team gave new direction to the reactivators of cholinesterase activity. The most well-known among them is HI-6, because the research so far shows that at the moment it is one of the best reactivators of the inhibited from Soman acetyl cholinesterase. As many countries have started using this drug, it is more and more important to learn its own toxicity and its pharmacological properties. Therefore, the aim of this study is to obtain a number of data, which characterize pharmacologically and toxicologically HI-6, which has been synthesized in Bulgaria.

STRUCTURE-ACTIVITY RELATIONSHIPS:

Oxime group at position 2 of the first pyridinium ring and the amide group in position 4 of the second piridinium ring are essential for the pharmacological activity against Ops (Maksimovic et al. 1980; Clement and Lockwood 1982). The 4 position in the second no-oximeted ring is particularly important both for the efficacy of HI-6, and other similar compounds, and for its inherent toxicity. An amide group at this position is essential for reducing the toxicity of the bispyridinium nucleus (Su et al. 1983, 1986).

In our research(Dishovsky, 1989,1996), just like Maksimovich in 1981, we have reached the conclusion that the correlation methods for analysis cannot explain the difference in the physicochemical properties of the reactivators. The theoretically calculated by us numbers of the distances between the atoms in a folded conformation, coincide well with the data from the Ro-structural research of Binenfeld,1984. The distance denoted with $d_{1\ to\ 4}$ are crucial for the biological activity of the oximes, particularly for the recovery of NMT blocked with OP (Figure 1).

(Figure 1) Structure of HI-6

TOXICITY OF HI-6:
The investigation of the acute toxicity of HI-6 of i.p. application on mouse has shown that it has lower toxicity in comparison with the other "H" -oximes, such as HS-3 and HS-6, as well as the classical toxogonin and 2-PAM. Our data shows to be analogical to the data of Kapner et al (1978).

Comparison of HI-6, produced in Bulgaria with other samples:
HI-6 was produced in the Military Medical Academy, Sofia with some differences from the method. described by Hagedorn. Clement et. al. (1988) compare produced under laboratory conditions and for commercial purposes substances of HI-6 from different countries. On (Table 1) is shown comparative data of the Bulgarian substances with substances produced in other countries.

(Table 1)

Comparison of HI-6 produced in different countries (Klement et. al 1988 and our data)

Country	Canada	UK	The Netherlands	Israel	Yugoslavia	Bulgaria
Type of substance	White crystal substance	Brown powder	Slightly yellow crystal substance	White powder	Yellow crystal substance	White Crystal Substance
Melting Point (^{0}C)	135-136 without impurities	132-134 with 3 additional picks	137-138,5 traces of impurities	174-175 without impurities	134-234,5 traces of impurities	135-137 without impurities
LD 50 mg/kg	561	495	563	-	658	586

The data, which was presented, shows that the Bulgarian HI-6 is close with its properties to the Canadian, the Dutch and the Yugoslavian substances. Of course, we should consider that the presented data is from research made in 1988.

Investigation of the blood pressure, breathing, and EKG of experimental animals, treated with HI-6:

The experiments were carried out on 33 anaesthetised cats (urethane) from both sexes, weighting 3.5± 1.0 kg. The cats were treated i.m. and i.v. with 20mg/kg b.w. We registered the researched animals breathing and arterial pressure under the McLeon method, 1970. We read EKG under standard conditions with EKG 111 (former DDR).

The conducted research showed that the i.m. application of HI-6 to cats does not cause changes of their arterial pressure, breathing and EKG. After i.v. injection the breathing becomes deeper for about 1 min.. after that it becomes normal again. The arterial pressure becomes 40-50 Hg lower for about 5 min. The EKG shows slowing down of the heart activity for about 5 min. Lundy and Tremblay (1979) suggested that HI-6 produse hypotension as a result of his ganglion – blocking activity.

Research of the effect of HI-6 of the spontaneous contraction activity of jejunum of a rat and during a contraction caused by Ach.

We have carried out the experiments on the jejunum of male Wistar rats, weighting 190± 30 g. HI-6 was used in increasing concentrations from 1.10^{-8} to 1.10^{-4} mol/ml. The results showed that HI-6, even in highest concentrations does not change the normal contraction activity of the gut pieces.

The spasmolitical activity of HI-6 is low. 100% removing of the effect of Ach (1.10^{-5} mol/ml) is made through concentration of HI-6 1.10^{-3} mol/ml.

Research of the HI-6 influence on the serum-ChE activity:

We have investigated the effect of the HI-6 on ChE activity during in vivo experiments on guinea pigs. b.w. 440± 60 g. We have applied HI-6 i.m in dosage 1.10^{-4} mol/kg. The results of the investigation are shown on the table below. It could be seen that HI-6 in the used concentration practically does not change for a period of 30 days the normal cholinesterase activity in the serum of the studied animals (Table 2)

(Table2)
Dynamics of the serum cholinesterase activity (E/ml, 25 ^{0}C) of guinea pigs treated with HI-6

Product/Drug	HI-6 1.10-4 mol/kg i.m.	
Time	X+- SD	P
Control	1616,4 ± 226,4	>0,05
1 day	1634,3 ± 244,1	>0,05
3 days	1607,1 ± 265,3	>0,05
10 days	1594,9 ± 292,9	>0,05
15 days	1618,9 ± 293,1	>0,05
20 days	1553 ± 248,7	>0,05
25 days	1523,1 ± 215,2	>0,05
30 days	1637,0 ± 225,4	>0,05

Pharmacokinetics of HI-6:

The pharmacokinetic data obtained with the Bulgarian HI-6 were presented on CBMTS II. (Dishovsky and Draganov,1996)The most important data was that the curve of the plasma concentrations of HI-6 ($0.5.10^{-4}$ mol/kg.) after i.v. application on cats until the 120 min. stayed the same above the Kusic et al (1985) 4 mkg/ml therapeutic concentration. A very important result is that the time of the semi-elimination is longer that the time of TMB-4 and toxogonine – 50,6 min vs. 28,3 and 19,9 min. respectively.

When the same dosage is applied i.m., the time for semi-elimination reaches 72,5 min. This is practically analogical with the data of Kusic et al obtained on human beings (1985). The results of the plasma clearance were – 3,1 vs 3,7 ml. min^{-1} kg^{-1}.
The data obtained for the plasma clearance shows that the substance passes quickly from the blood into the organs. The seemingly large volume points to the conclusion that there is a large amount free HI-6 in the plasma.

Biochemical and hematological research of HI-6:
We researched ten dogs of both sexes with b.w. 9 ± 1,5 kg. We have applied HI-6 in dosage 6 mg/kg i.m. We have made the observations on the 2nd, 24th and 48th hour.
We studied GOT, GPT (ALT), Alk. Phosphatase, LDH, blood sugar, serum protein, thymol, creatinine, bilirubin, urea, calcium and natrium. We analyzed from chematological point of view the Erythrocytes, Leukocytes, Lymphocytes, Polinuclear cells and Thrombocytes. We have not found any significant statistical deviations from the norms.
The obtained data after application of HI-6 does not show statistically reliable reactions. This is a proof that HI-6 does not cause toxic and unwanted reactions toward the biological and hematological indexes.

Acid-base balance
We made the research on dogs with b.w. 9,5 ± 1,5 kg (n=6). We applied HI-6 i.m. with dosage 3 mg/kg. The researched parameters were pO_2, pCO_2, pH, BE, HCO_3, SB and BB. (The research was conducted by Dr. I. Sumnalev on AVL equipment).
We made observation for certain decrease of pCO_2, which presents an alveoli hyper-ventilation and decrease of HCO_3, which is an indicator of respiratory alkalosis. However, the pH stays the same all the time during the experiment. As a whole after the application of HI-6 there are no statistically considerable deviations in the researched parameters.

Histo-morphologic investigations:
The research was conducted on 25 Wister rats with b.w. 190 ± 30 g. In a cute experiment 5 animals were injected i.m. with 5 mg./kg HI-6 and the observation was made on the 2nd hour. The rest of the animals were distributed in groups of 5 as follows:

- getting i.m. 1 mg/kg HI-6 and observed on the 15th day;
- getting i.m. 5 mg/kg HI-6 and observed on the 15th day;
- getting i.m. 1 mg/kg HI-6 and observed on the 30th day;
- getting i.m. 5 mg/kg HI-6 and observed on the 30th day;

The following body organs were analysed: brain, miocardium, lung, v. cava, liver, kidney and skeleton mussels in the place of injection. The method of hematoxilin-eozin was used. During the histo-morphologic investigation were detected changes of the investigated organs due to the use of HI-6.

Analysis of the HI-6 influence on the liver drug metabolizing enzyme systems:
The reactivators of the Che activity are almost always used with other drugs. Our previous research showed that toxogonine change the liver drug metabolizing enzime systems. Together with Dr. Kadiiska and P. Alov , we conducted investigations on Wister white male rats with b.w. 180 – 200 g. (n=150). We made the analysis on the 2nd and 4th hour with HI-6 with dosage 0,25mmol/kg i.m., as well as after three-day application of the same dosage.
Cytochrom P-450 and cytochrom b5 contens in microsomes werte determined by the method of Omura and Sato (1964); ethylmorphine-N-demethylase (EMND) by
Nash(1953); aniline 4 - hydroxilase (AH) by Mazel (1971); glucuronyl transferase (GT) by Frei (1970). Protein content in microsomes was determined by modified Lowry procedure (Albro, 1975). Lipide peroxidation in liver microsomes was determined by thiobarbituric acide test of Buege and Aust 91978).

From the obtained data, it could be seen that HI-6 shortens the time of the hexobarbital sleep in the interval 2nd hour. The data shows that in this dosage HI-6 does not change the liver drug metabolizing enzymes and the LPO.

CONCLUSION

The data obtained by us, is very important as support for the further discussion on the liver changes caused by HI-6 (Kusic et al, 1991) for analysis on human beings. We have not denoted any changes in the researched indicators, apart from some decrease of the thymol and bilirubin does not necessarily mean that there is a toxic damage of the liver.

In previous our research (Dishovsky, 1990, 96), we have found changes in the pharmacokinetics of HI-6. Considering that HI-6 under normal conditions, will not be used for a long time, we think that it has to be accepted as a perspective reactivator of the Che activity. A proof for that are all the results, which we have obtained, and that we present in the above paper.

It is recommendable, if possible, to follow up the serum concentration of HI-6, as well as the para-clinical data, which will allow for the optimization of it clinical effect in cases of intoxication with OPC.

KEYWORDS: HI-6, H-series oximes, cholinesterase, pharmacology, toxicology

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6. PLANNED RESEARCH TO EXTEND AND FURTHER VALIDATE RSDL AS A BROAD SPECTRUM DECONTAMINANT AGAINST BIOLOGICAL AND CHEMICAL WARFARE AGENTS

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SUMMARY OF RECENT DEVELOPMENTS

Reactive Skin Decontamination Lotion - RSDL - is now fielded with the Canadian Forces, the Netherlands Armed Forces, and several other countries and organizations worldwide. It is also in use in several Chemical Warfare Defense laboratories for the safety of the researchers. Knowledge of RSDL and its capabilities, recently further verified by the reports of the TNO Prins Maurits Lab, the German Government, and the French DGA, RSDL is being requested increasingly for both military and civil defense applications including VIP visits.

All of this, combined with new and evolving threats, has provided a need to determine exactly how and to what extent RSDL may be used as a protective means against this increased range of Chemical and Biological Warfare (CBW) materials and situations.

In support of these increasing requests, both from inside and outside of the traditional military arena, a number of thrusts are underway to provide full support and information to users of RSDL in order that they understand the capabilities and limitations of the material when it is used in the field.

The human compatible nature of RSDL and its extremely rapid reaction time with typical CW agents has made it an ideal candidate for use in cleaning and/or leak mitigating when handling chemical weapons discovered in the field. Use of RSDL in this application will hopefully will make this high-risk operation somewhat safer for the personnel directly involved.

In addition, the nature of the reaction of RSDL when removing and destroying chemical agents has led to its being considered for equipment decontamination of sensitive, high value items. The rapid action, lack of temperature build up, and easy removal make RSDL suitable for cleaning many types of equipment very quickly.

Another area of use, which was found by the Canadian Forces, is the ability of the RSDL system to remove irritants from the skin. This specifically applies to removing riot control agents such as OC (Pepper Spray) and CS.

All of these areas require controlled and systematic examination and it is planned to address them this year and in 2001. This will parallel the Regulatory review which is being done to support the full licensing of RSDL for use in Canada by and on civilian victims of CBW events as well as to fully assure military personnel of the safety of the products they are being issued as a defense against these threats.

INTRODUCTION

Reactive Skin Decontamination Lotion - RSDL - was developed by the Defense Research Establishments Ottawa and Suffield (DREO and DRES) of the Canadian Department of National Defense (DND) as a general issue first line decontaminating liquid to combat a wide spectrum of Military Chemical Weapons. RSDL was specifically developed to remove, from any part of the body, and render non-toxic the full spectrum of nerve agents and vesicants that could potentially be encountered by Canadian troops in any conflict or other operation worldwide.

The nature of RSDL as a liquid decontaminating lotion was driven by these requirements. RSDL is a liquid to provide two key advantages: First, for full and complete surface coverage on skin, through permeable clothing, and through hair, and second, to enable the active component of the lotion to easily mix with any chemical agent present and rapidly destroy it.

RSDL has been formulated and tested[1] to allow its use as a topical decontaminant on skin, in the eyes, and in and around the mouth and ears. The RSDL system covers and lifts an agent from the skin and into solution. The active ingredient then breaks down the agent to provide a non-toxic[2] liquid residue, which does not need to be immediately removed from the skin. The liquid RSDL can be left in place to provide a better mask seal and continuing decontamination capacity. RSDL minimizes the time and effort required to provide self aid in an urgent situation where

other threats may be present. The material can be easily washed off with water later during full personal decontamination.

APPLICATIONS OF RSDL IN LEAK SEAL AND PACKAGING OF OCW MUNITIONS

RSDL has a number of characteristics which make it suitable to be considered for use in cleaning and preparing for transport, chemical munitions discovered in the field and which for local reasons may not be destroyed in situ. RSDL will rapidly mix with and destroy a wide range of agents and the lotion in the sponge system will remain in contact with the subject munitions providing continuing destruction of any material that is subsequently released.

Color

The lotion color indicates whether the agent has been fully destroyed or whether active agent remains present. RSDL is naturally a very bright yellow color and as the active ingredient is consumed in breaking down chemical agents the yellow fades to a clear-white solution when full consumption of the active ingredient occurs. Should this occur, additional RSDL can be added until the yellow color remains, indicating that the agent present at that time has been destroyed.

Rapid Action

If, as a munition is uncovered or during immediate recovery it begins to leak, RSDL can be rapidly and easily applied directly on the leaking liquid. The sponge can be used to cover the leak to provide some level of seal and immediate destruction of leaking agent until a full sealed package can be brought in or applied. Excess RSDL can be included in the package to provide protection and/or agent destruction during transport.

Human Compatibility

A highly dangerous part of recovering munitions in the field is in actually removing them from their present location to a place where they can be examined and worked on or destroyed. This may entail simple excavation, a trip of a few meters, or lengthy transport, but the first exposure in many cases will involve personnel being in close proximity to the munition. Often actual physical handling may be required and using RSDL to provide immediate decontamination of the munition increases the safety for the operators present as RSDL will not react aggressively with the agent nor will it react against the gloves and boots[1] as a more caustic material might. Any reacted products present will be non-toxic[2] and will not harm an operator.

EQUIPMENT DECONTAMINATION USING RSDL

RSDL is fully compatible with the skin and thus any equipment that is essentially equally durable can be decontaminated with RSDL. Specifically, typically finished metal surfaces (anodized, phosphated, painted, etc.) on military equipment can be decontaminated with RSDL. For example, vehicle hatches and handles can be rapidly decontaminated to provide access to the vehicle or to allow safe egress of passengers in cases where they can not wait for the vehicle to be fully decontaminated before exiting.

Another area of equipment decontamination, which has recently been explored, involves cleaning high value equipment such as tactical radios, night vision devices, Global Positioning System receivers, and the like. This equipment is generally ruggedized for military use, sealed to prevent ingress of environmental effects (water) to the operating hardware, and has one or more display units, from which the operator reads the information. This equipment is generally valuable, it can not be readily replaced in a theatre of operations, and loss of it can often significantly and immediately degrade operational capability.

Our testing to date has shown RSDL can be applied to military equipment of this type and removed using water. The equipment was not adversely effected in any way. The equipment continued to operate correctly, displays functioned and were legible, markings were not obscured or removed, and seals were not damaged or breached.

Testing using uncontaminated equipment as a baseline and using BW simulants have shown promising results for compatibility with the lotion and for the effective and efficient removal of the simulant. Further testing against actual CW and BW agents to determine the useful ability of RSDL to remove them from typical equipment surfaces is planned.

RSDL FOR THE REMOVAL OF RIOT CONTROL AGENTS OC AND CS

The Canadian Forces have been using the RSDL system to remove CS from personnel during training for approximately one year. A short series of demonstrationshas shown that the RSDL system can be used to ease the effectsof and remove OC from skin and, in one case, from the eyes.

In practice, the use of RSDL to remove these riot control agents would be completed after the incident requiring the application of the OC or CS had been resolved. In particular, Police Officers or other emergency services personnel would use the RSDL system to remove any OC or CS spray which had contacted them either directly from the spray device or from subsequently handling suspects who had the spray applied to them. Suspects suffering from the effects of the OC or CS could, once they were calmed or in custody, have this applied to ease their situation and to clear their eyes. It could also be used in training to minimize the effects of the OC or CS spray when used in teaching new personnel or in refresher training.

It is planned to test the effectiveness of the removal of OC and CS using a model which can be quantitatively evaluated against other methods to validate whether the use of RSDL is equal to, worse than, or superior to other available methods of OC and CS removal or mitigation.

EVALUATING RSDL EFFECTIVENESS AGAINST BW AGENTS

RSDL was originally developed as a system for the removal and destruction of CW agents. Increasingly, we are asked about its usefulness and effectiveness against Biological Warfare agents. Two tests carried out in the last two years have indicated that the presence of RSDL may have positive effects in inhibiting or destroying some BW agents.

It was shown that some BW agent simulants could be destroyed by contact with RSDL. Other BW agent simulants were not effected by the presence of RSDL but did appear to be taken into solution where they could at least be controlled and removed.

It is our intent, based on these results, to further study the effects of RSDL on actual BW agents including bacteria, viruses, toxins and spores to determine to what extent the presence of RSDL either destroys or inhibits their actions, and further, to determine how effective RSDL is in lifting and removing various types of agents for which it does not provide reasonably rapid destruction. The usefulness of RSDL versus other typical means will be considered to determine whether RSDL provides a significant increase in performance versus other means of BW agent removal.

Current use of RSDL for BW agent containment within the CASCAD-COLPRO[3] system would contain and destroy all biological agents through the immediate use of RSDL to contain the contacted agent preventing inhalation, the use of the shower system for removal of RSDL and any remaining live agent and finally the residual agent would be destroyed by the reuse of the shower water as part of the vehicle decontamination process. Application of the CASCAD chemicals would ensure the rapid and full destruction of any remaining BW agent materials in the source water, part of which would be from the shower system.

CURRENT STATE OF REGULATORY REVIEW IN CANADA

The Canadian Forces made a decision to review and present their CW and BW defense materials to the Canadian federal health regulatory body, Health Canada (HC), for licensing for use in the same manner as civilian developed drugs, medical devices, etc. under the Federal Government medical controls system.

RSDL, as the first line personal decontamination system for the entire Canadian Forces, is therefore being put through this process. This means that all of the data gathered to date regarding formula, efficacy, safety, stability, and production is being reviewed and scrutinized in order to be submitted as part of the licensing request (New Drug Submission). In some cases the data is having to be recreated and this is causing some concerns as this redevelopment and retesting may be seen to imply that previous product was not manufactured or tested as rigorously as possible. This is not the case, but it is necessary to ensure that the process is completed exactly as required by HC in order to be eligible for the issuance of a Drug Identification Number (DIN) at the conclusion of the review.

In addition, a few areas of "normal" testing which are required by the regulatory process, specifically those relating to the testing of the active ingredient (drug substance), are rather unique when considering RSDL. The key active material used is novel and unique, and thus most test methods required need to be developed or extensively validated prior to their acceptance by health authorities. This adds considerable time and cost to the process. In addition, RSDL and its active ingredients cannot be tested in an aqueous system. The typical test methods, which assume aqueous solubility for testing, cannot be used, adding further difficulty and complication. This is a key area to consider with materials such as this since any proposed test method must be fully validated and accepted by the governing Health Authorities.

The manufacturing aspects of any medical or drug related licensing submission are extensive, but as RSDL has always been produced by the current licensee in a pharmaceutical grade facility, this is well under control. Had the

production not been completed in this type of facility a number of problems including the extensive traceability of produced lots, the batch and packaging records, and the qualification of pharmaceutical grade suppliers would not necessarily have occurred, and certainly would not have been done as quickly.

Finally the testing of this type of product in clinical study is severely limited as the typical "double blind" test protocol would result in some very adverse reactions for one quarter of the test subjects. Thus it is necessary to use the other developmental tests and simulation to demonstrate the necessity of and efficacy of the product. Clinical testing will be limited to the "null case" or the application of the candidate material to an unaffected person - essentially skin sensitivity testing - once the initial review of the existing lab tests is completed and the Health Authorities have given permission to go ahead.

It is hoped that the remaining testing will be completed within the next 18 months so that an application for a DIN can then be submitted.

ACKNOWLEDGEMENTS

The support of the Canadian Department of National Defense through the Directorate of Health Operations and the Defense Scientists of the Defense Research Establishment Suffield cannot be understated. They have been key in providing expert knowledge and support of the commercialization of the process and in contact with Health Canada to help in completing the registration process. The entire process of taking this research into a real world product has been a tight cooperative effort between Industry and the Canadian Forces throughout and would not have been possible without the support of key personnel in these agencies. Only through this continuing close cooperation will the successful completion of the process be possible.

We would like to formally acknowledge and thank DND and particularly Defense Research Establishment Suffield for this support as we go forward on this project together.

NOTES

1. All testing referenced has been completed by the Canadian Forces, by, or on behalf of other Governments.
2. Agents such as Lewisite, which contains Arsenic, will be broken down but the Arsenic must be considered at the decontamination station as it will remain, in a less toxic form, in the lotion residue.
3. **CASCAD-COLPRO**: **CA**nadian **S**ystem for **C**hemical-biological **A**gent **D**econtamination - **COL**lective **PRO**tection enclosure

Rational Design of Botulinus Neurotoxin Therapies

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Short Term Goal : Discover novel inhibitors of Botulinus neurotoxin A
Long Term Goal : Design oral inhibitors of Botulinus neurotoxin A

BACKGROUND :
Botulinus neurotoxin A (Botox A) is one of several protein toxins from *clostridium botulinum* which cause paralytic syndromes resulting from blockade of neurotransmitter release. These toxins are all zinc endopeptidases acting in the neuronal cytosol: Botox B, D, F and G as well as tetanus toxin attack specifically VAMP (also called synaptobrevin) – a protein of synaptic vesicles; Botox A and E cleave SNAP-25 and Botox C acts on syntaxin – both proteins of the presynaptic membrane

Botox A is a 150K protein made up of a 50K light chain (amino acids 1-448) and a 100K heavy chain (amino acids 449-1296) which are held together by a disulfide bond (C430-C454). The heavy chain contains a transmembrane domain (residues 659-681) which inserts into the neuronal cell membrane and enables the light chain to access the interior of the neuron. Intracellular reduction of the interchain disulfide activates the proteolytic activity of the light chain (zinc-binding domain HELIH, residues 223-227) which then cleaves the protein SNAP-25 and disables the docking mechanism required for exocytosis of neurotransmitter.

SNAP-25 is one component of the so-called SNARE complex which is responsible for docking synaptic vesicles and fusion to the cell membrane as the immediate precursor event to transmitter release. The SNARE complex is a four-helix bundle made up of two small proteins, namely VAMP and syntaxin, and a larger protein SNAP-25 which doubles back on itself and provides two of the four threads of the helix bundle. SNAP-25 also contains a lipid anchor region (amino acids 85-92) between the two helical threads, which lines up with transmembrane domains at the C-termini of both VAMP and syntaxin (Poirier et al. Nature Structural Biology 5:765,1998).. Whereas Botox A selectively cleaves the Gln187-Arg203 bond near the C-terminus of SNAP-25, the other botulinus neurotoxins each cleave selectively a different peptide bond within one of the three target proteins which comprise the SNARE complex.

A repeating motif exists within the sequences of all three of the proteins of the SNARE complex which, when introduced in the form of synthetic peptides of about ten residues in length, inhibits the actions of botulinus toxins on the SNARE complex (Rossetto et al, Nature 372: 416, 1994). For example one version of the repeating motif present in VAMP, designated V2 and having the sequence 62ELDDRADALQ71, blocks the neurotoxic actions of Botox A and B when the peptide is injected into cultured Aplysia neurons. The significance of the repeating SNARE motif, which appears twice on each of the four threads of the SNARE helix bundle upstream of the cleavage sites, is not well understood, although it presumably acts as a recognition site for binding of some other biomolecule(s), and may also be used by Botox as a binding-recognition element. In agreement with this, cross recognition of the target proteins by the various toxins occurs: Botox A inhibits VAMP proteolysis by Botox B, and Botox B and tetanus inhibit the cleavage of SNAP-25 by Botox A. Moreover serum albumin, which contains SNARE motifs within helical regions of its secondary structure (183DELRD187 and 255DDRAD259), inhibits the cleavage of synthetic substrate by Botox A.

Thus although the mechanism of action of V2 (and other variants of the SNARE motif) is not proven, it could involve binding to a complementary recognition site on Botox resulting in inhibition of productive binding of Botox to the SNARE complex. Interestingly, possible complementary SNARE motifs (592KKVNK596 and 701KRNEK705 in the heavy chain, and 335KLKFDK340 and 359KVLNRK364 in the light chain) exist in Botox A.

STRATEGY :

Several possible strategies for inhibiting Botox exist:
block the transmembrane domain of Botox and prevent cell entry, e.g. complementary peptides, antibodies;
block the proteolytic site of Botox, e.g. substrate inhibitors, selective chelation of zinc;
block the interaction of Botox with binding-recognition motif, e.g. mimics of SNARE motif which bind to complementary site(s) on Botox.

The first strategy has potential application prior to exposure to toxin (e.g. immunization), as well as for the deactivation of toxin in body fluids after exposure. The latter strategies, on the other hand, have the potential for providing total body treatment after toxin exposure - assuming that the therapeutic agent is able to cross cell membranes and inactivate toxin which has already entered the cell. Whereas agents based on active site inhibitors will have to be tailored to individual variants of Botox, therapies based on the SNARE motif have the potential to treat poisoning by all forms of Botox as well as tetanus.

Active sites of zinc proteases:

The most widely studied of all the zinc proteases is carboxypeptidase A (CPA), and information on the mechanism of action of this enzyme serves a useful basis for understanding other related zinc enzymes such as Botox.

Detailed X-ray studies on CPA have illustrated that the tetradentate zinc atom sequesters a single water molecule and is tethered by coordination to the imidazole groups of His-69 and His-196 as well as to the carboxylate of Glu-270. CPA undergoes a conformational change on binding of the substrate Gly-Tyr in which the guanidinium group of Arg-145 moves 2A to form a salt bridge with the C-terminus of the substrate, the carboxyl group of Glu-270 moves 2A away from the zinc atom and forms a salt bridge with the amino group of the substrate (can only occur with dipeptide), and the phenolic group of Tyr-248 moves 12A to within 3A of the scissile bond (Reeke et al, PNAS 57, 2220, 1967). Chemical modification studies have suggested that Tyr-248 is essential for the peptidase activity, but not the esterase activity, of CPA. A catalytic mechanism has been proposed in which the susceptible carbonyl oxygen of the substrate coordinates to zinc and the Glu-270 carboxylate attacks the carbonyl carbon to form a mixed anhydride intermediate, which is subsequently hydrolyzed by a base catalyzed mechanism involving the nascent water molecule (Zn-OH).

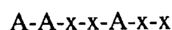
The best inhibitors of CPA are generally compounds which coordinate to the active site zinc and either sequester the zinc atom away from the enzyme or prevent access of the substrate to the catalytic centre. These include thiols, amines/imines, flavonoids, and a range of anionic groups including carboxylates, phosphonates, etc.The most potent inhibitors will be those having an exact fit to the active site of the enzyme which is accompanied by strong attractive forces resulting in high affinity binding not only to the zinc atom but also to the enzyme's peptidic groups.

Due to its preference for basic amino acid residues, the enzyme most like Botox A is carboxypeptidase B. CPB differs from CPA in that it contains three disulfides (CPA has only one) and the coordination of zinc involves a free Cys thiol. Exchange of zinc for cadmium in CPB results in only esterase activity (no peptidase activity). Replacement of Asp-253 (which normally binds the basic sidechain of the substrate) with Lys gave a reversed-polarity mutant of human CPB which hydrolyzed hippuryl-L-glutamic acid (Edge et al, Protein Eng. 11, 1229, 1998).

The best inhibitors of CPA are benzyl- or alkyl-succinic acids and, assuming some parity between CPA and CPB, likely inhibitors of CPB would be aminoalkyl- or guanidinoalkyl-succinic acids. For reasons of specificity, it follows that those considerations which apply to CPB will likely also apply to Botox A. Accordingly, peptide libraries directed towards the active site of Botox A should be rich in basic and acidic moieties. Interestingly, acidic residues are also a characteristic of the SNARE motif, so that peptide libraries composed of these residues should interact with both the catalytic site and the SNARE motif site and act as dual inhibitors.

Regardless of mechanism, one approach to producing improved inhibitors of Botox is to identify the structural elements of V2 (and other SNARE motifs) which make it an effective inhibitor and then reconstruct these components into a smaller and preferably nonpeptidic molecule which would be able to traverse membanes, thereby providing not only access to the inside of cells but also the potential for oral activity. A streamlined and efficient approach to this goal is to create a library of small semimimetic peptides containing the essential elements of the repeating motif in the SNARE complex, identify the most active peptide in the mixture by iterative deconvolution of the library, and then restructure the best semimimetic peptide identified into a fully fledged nonpeptide mimetic using computer molecular modelling techniques.

The important structural feature of the SNARE motif is comprised of an amino acid sequence made up of residues:



A-A-x-x-A-x-x

where A=acidic residue and x=nonpolar or polar residue.

Due to the helical arrangement of these groups within the secondary structure of the SNARE proteins from which they are derived, the end result is a cluster of three neighbouring negative charges juxtaposed by a nonpolar moiety. In other words, the required motif can be envisaged as three negatively charged groups mounted in close proximity on a hydrophobic template. The simplest representation of this which comes to mind is a benzene ring with three carboxymethyl groups attached at the 1, 3 and 5 positions. Other variations on this theme can also be easily envisaged, and a number of commercially available compounds which fit this general scheme are being investigated for inhibitory activity in our laboratory, together with several peptide libraries which have been designed and synthesized with these considerations in mind.

Design and Synthesis of Peptide Libraries:

One library has been synthesized which is designed to mimic the SNARE motif:

SN-1 = Ac -X1-X2-LINKER-X3-X4-NH2,

where X1, X2, X3 & X4 are mixtures of Asp, Glu, Gln and Arg,
and the LINKER group is 4-aminobutyric acid

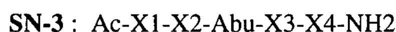
The rationale for the design of the peptide minilibrary SN-1 is based on the preponderence of acidic residues in SNARE motifs, together with the occurrence of other residues in SNARE motifs which can provide solubility properties to the libraries – hence the inclusion of Arg and Gln in SN-1. The multiple negative charges represented by the Asp and Glu residues, when mounted around the flexible linker group 4-aminobutyric acid (Abu), should engender structural and conformational variations on the SNARE motif which will inhibit Botox

Coincidentally the Gln and Arg residues in SN-1 are also representative of the scissile bond (Q-R) in the Botox A substrate, so that the peptide minilibrary SN-1 doubles in a rudimentary way as a substrate mimic. This parallels the classical approach to inhibitor discovery, which is usually done by minor modification of substrate structure leading to compounds which bind to the active site but are not cleaved by the enzyme. Accordingly, incorporating residues of the scissile bond (Gln-Arg) into peptide libraries such as SN-1 should provide structural and conformational characteristics which might result in substrate-based inhibitors. Clearly SN-1 contains the potential for containing inhibitors based not only on the SNARE motif but also on substrate structure.

Another consideration for the design of peptide libraries with inhibitory potency is the inclusion of residues which would be expected to coordinate to the active site zinc atom of Botox, namely Cys and His as well as acidic residues. The approach here is to create a peptide library containing an array of zinc binding

elements in a variety of different formats, wherein there should be individual peptides which recognise not only the zinc atom but also unique aspects of the active site of Botox A in which the zinc atom resides. The intent is to produce zinc targeted inhibitors which show selectivity for Botox over other zinc metalloenzymes.

An example of a library targeted to produce active site zinc inhibitors is:-


SN-3 : Ac-X1-X2-Abu-X3-X4-NH2

where Ac = acetyl, Abu = 4-aminobutyric acid, and X1, X2,. X3, and X4 are mixtures of the amino acids Asp, Glu, His and Cys.

This library contains both active site zinc directed probes *and* variations on the SNARE motif within the same basis set (library) of peptides.

Conformational properties of target binding sites:

Before intricate design work on inhibitors of Botox can be undertaken, details of the three dimensional conformation of the target sequence must be elucidated. In the case of Botox A, the substrate SNAP-25 is believed to form part of a four helix protein bundle which is anchored into the presynaptic membrane. Actually the cleavage site targeted by Botox A is near the C-terminus of SNAP-25, which is far removed from the the central lipid anchor region of SNAP-25. Similarly the amino acid sequences representing the four SNARE motifs of SNAP-25 (S1, S2, S3 and S4) reside in the central part of the SNARE complex away from the lipid anchor domain found at one end of the tubular bundle. Accordingly, it is not clear if the environment surrounding the sequences representing not only the cleavage site but also the SNARE motifs is primarily polar (aqueous) or nonpolar (membrane lipid) in character.

As a precautionary measure we have conducted conformational calculations on these target sequences firstly in a nonpolar environment and subsequently in a polar environment. Semiempirical energy minimization calculations carried out on the substrate SNAP-25 (187-203) revealed that this peptide takes up a helical conformation in a nonpolar environment (in vacuo), but that the introduction of water molecules into the environment results in unravelling of the helix. Accordingly the conformation of this peptide is highly dependent on environment, although the spatial arrangement of amino acid sidechains in the critical region of the QR scissile peptide bond remains the same. It is not clear if Botox A is likely to preferentially recognize either the helical conformation or the disrupted conformation, and at present there is no way of knowing which of the two conformations would be appropriate for design work on potential active site inhibitors.

Likewise, semiempirical calculations on the SNARE motifs V2 and S3 have revealed helical structures in a nonpolar environment which become disrupted in the presence of water. Again, since there is no way of knowing which is the biologically relevant conformation, it is not possible to conduct design work based on the information presently available. Consequently we have elected to proceed with peptide library investigations in the hope that the identification of a bioactive semimimetic peptide will provide the answer to this question. Thus, eventually it should be possible to identify from library screening, a potent inhibitory ligand containing the essential structural elements of the SNARE motif, which is sufficiently conformationallly restricted that it can only take up one of the two SNARE motif conformations – either that found in membranes or that found in water. At that stage it will then be possible to use the appropriate conformational model for further detailed design work.

Optimization of assay conditions:

Several laboratories have reported assays of Botox A activity against various substrates. Rossetto et al (1994) investigated the action of Botox A against intact SNAP-25 and found that only the reduced form of the enzyme (pretreated with 10mM DTT for 30 min at 37C) could cleave the substrate. For the subsequent incubation with substrate, they used 5mM HEPES buffer pH 7.4 for 60 min at 37C, without the addition of

supplementary zinc. These authors also reported that the enzyme was blocked by 10mM EDTA (zinc chelator) or by 1.4mM captopril (zinc endopeptidase inhibitor).

Schmidt & Bostian (1997) studied the activity of Botox A towards a number of short synthetic substrates derived from the cleavage site of SNAP-25 and found that peptides of at least 15 residues were required for cleavage by the reduced form of the enzyme. Incubations were carried out in 30mM HEPES buffer pH 7.3 in the presence of 5mM DTT, 250uM zinc and 1mg/ml BSA for 10min at 37C. Activity against these substrates required the presence of BSA and increased with increasing concentrations of BSA to a maximum effect at 1mg/ml BSA. The activity was blocked by 5mM EDTA or 100mM Tris (zinc chelators).

In this laboratory we have investigated the effects of pH and varying concentrations of zinc and DTT on the activity of the reduced enzyme. Reduction was accomplished by incubating the enzyme with 20mM fresh DTT at 37° for 30min in 50mM HEPES pH 8. After reduction optimal activity against a 17mer peptide from SNAP-25(residues 187-203) was observed by preincubating the enzyme (5nM) in 15mM HEPES buffer pH 6.9 in the presence of 1uM zinc and 0.5mM DTT for 5 hr before addition of the substrate (50uM). After 30min the reaction was terminated with an equal volume of 1% TFA and the products determined by HPLC. The activity was inhibited in the complete absence of zinc or in the presence of zinc concentrations above 10uM, and was also inhibited by 10mM DTT. These findings illustrate that Botox A, like many other zinc endopeptidases, requires the presence of zinc but is inhibited by excess zinc. In addition, since high concentrations of DTT can inhibit, possibly due to the zinc chelating properties of DTT, a careful balance between zinc and DTT concentrations must be engendered.

Inhibitors of Botox A:

In our studies a number of compounds were found to inhibit the Botox A mediated cleavage of the 17-amino acid synthetic peptide substrate
Ac-SNKTRIDQANQRATKML-NH2, which derives from the C-terminal part (residues 187-203) of SNAP-25 and contains the scissile QR peptide bond targeted by Botox A (Table 1). Several thiol-containing compounds, namely dithiothreitol (widely used to reduce disulphide bonds in proteins), DMPS (a potent chelator of heavy metals) and Captopril (a clinical inhibitor of the zinc dipeptidase Angiotensin Converting Enzyme) block the cleavage of this substrate. However the non-thiol ACE inhibitor Lisinopril was ineffective, whereas the non-thiol prodrug ACE inhibitor enalapril was an effective inhibitor. The thiol compounds DMPS, captopril and DTT presumably act by a mechanism involving sequestration of the zinc atom at the active site of Botox A. The inhibitory activity of enalapril was unexpected but could suggest an affinity for the active site of Botox A which is not shared by lisinopril.

The synthetic peptide V2 was also able to inhibit Botox A, but at a higher concentration (5mM) than has been observed previously in vivo when SNAP-25 was the substrate (100uM, see Rossetto et al 1994). This may reflect the absence of SNARE motif in the short synthetic substrate used for the present studies.

Interestingly serum albumin (10uM), which contains the SNARE motif in duplicate, inhibited cleavage of SNAP-25(187-203) under our assay conditions which had been optimized for zinc (1uM) and DTT (0.5mM) concentrations. In contrast Schmidt & Bostian (J.Protein Chem. 16: 19, 1997) observed that in the presence of 250uM zinc and 5mM DTT cleavage of this substrate by Botox A only occurred in the presence of BSA; our findings would suggest that this was probably due to sequestration of inhibitory levels of zinc/DTT by BSA.

Investigations of SN-1 on the Botox A cleavage of substrate have shown that this peptide minilibrary does inhibit the enzyme activity (Table 1). This suggests that variations on the SNARE motif present in the library mixture may interfere with binding of Botox to the substrate. However a series of peptides which were selected for their potential to represent simple variations on the SNARE motif, i.e. Glu-Glu, Glu-Glu-Leu, Glu-Glu-Glu and Glu-Pro-Glu-Thr, were generally inactive (data not shown), with the notable exception of Glu-Glu-Glu. A number of peptides with potential complementary sequences of the SNARE motif, i.e. Lys-Arg, Lys-Lys, Orn-Orn, Lys-Lys-Lys, Orn-Orn-Orn and Lys-Phe-Gly-Lys, were inactive as expected because the synthetic 17mer peptide substrate used for the assay lacks the

repeating SNARE motifs that are present in the longer SNAP-25 natural substrate. However these basic peptides would be expected to inhibit the cleavage of SNARE proteins by Botox enzymes in general, if they bind well to the SNARE recognition motifs in these proteins.

Interestingly glycyrrhizic acid, which is a steroid glycoside containing 3 carboxylate groups, was able to inhibit Botox (Table 1). This finding, as with Glu-Glu-Glu, suggests that certain configurations of negative charges are able to approximate the SNARE motif and inhibit Botox A. However in the final analysis it would appear that the most potent inhibitor of Botox containing a SNARE motif variation is likely to be found in the peptide library SN-1 because each peptide in the mixture was present at 20uM concentration (Table 1).

Finally the present experimental findings also indicate that the best inhibitors of Botox A are likely to be derived from thiol containing compounds (the inhibitory activity of DMPS is particularly remarkable). In agreement with this, the peptide minilibrary SN-3 turned out to be an exceptionally potent inhibitor of Botox A (Table 1). When the overall thiol concentrations of the inhibitors are compared (Table 1) it is apparent that the peptide library SN-3 is the most potent thiol inhibitor of Botox. Furthermore it is very likely that SN-3 contains a peptide(s) which is a more potent inhibitor than DMPS.

We are currently evaluating SN-3 inhibition of other related zinc protease neurotoxins namely Botulus B and tetanus neurotoxin. The intent is to deconvolute the library and determine which peptide in the mixture is the best inhibitor of Botox A and which peptide in the library is the best inhibitor of Botox B and tetanus. The expectation is that there will be sufficient differences in the active sites of the toxins to yield different optimal inhibitors from the same 'hinge' peptide minilibrary.

ACKNOWLEDGEMENTS:
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Table 1. Inhibition of Botox

Inhibitor	Concentration (mM)	% Inhibition*	-SH Concentration (mM)
Dithiothreitol	1	26	2
DMPS‡	0.1	72	0.2
Captopril	1	76	1
Lysinopril	5	7	0

Enalapril	5	40	0
V2 (ELDDRADALQ)	5	40	0
Glu-Glu-Glu	5	22	0
Glycyrrizic acid	5	55	0
Library SN-1	5 (20 μM)[†]	16	0
Library SN-3	0.5 (2 μM)[†]	51	0.125

8. RATIONAL DESIGN OF BOTULINUS NEUROTOXIN THERAPIES

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BOTULINUS NEUROTOXIN INHIBITORS

Short Term Goal: Discover novel inhibitors of Botulinus neurotoxin A
Long Term Goal: Design oral inhibitors of Botulinus neurotoxin A

BACKGROUND

Botulinus neurotoxin A (Botox A) is one of several protein toxins from *clostridium botulinum,* which cause paralytic syndromes resulting from the blockage of neurotransmitter release. These toxins are all zinc endopeptidases acting in the neuronal cytosol: Botox B, D, F and G as well as tetanus toxin attack specifically VAMP (also called synaptobrevin) – a protein of synaptic vesicles; Botox A and E cleave SNAP-25 and Botox C acts on syntaxin – both proteins of the presynaptic membrane

Botox A is a 150K protein made up of a 50K light chain (amino acids 1-448) and a 100K heavy chain (amino acids 449-1296) which are held together by a disulfide bond (C430-C454). The heavy chain contains a transmembrane domain (residues 659-681) which inserts into the neuronal cell membrane and enables the light chain to access the interior of the neuron. Intracellular reduction of the interchain disulfide activates the proteolytic activity of the light chain (zinc-binding domain HELIH, residues 223-227) which then cleaves the protein SNAP-25 and disables the docking mechanism required for exocytosis of the neurotransmitter.

SNAP-25 is one component of the so-called SNARE complex, which is responsible for docking synaptic vesicles and fusion to the cell membrane as the immediate precursor event to transmitter release. The SNARE complex is a four-helix bundle made up of two small proteins, namely VAMP and syntaxin, and a larger protein SNAP-25 which doubles back on itself and provides two of the four threads of the helix bundle. SNAP-25 also contains a lipid anchor region (amino acids 85-92) between the two helical threads, which lines up with transmembrane domains at the C-termini of both VAMP and syntaxin (Poirier et al, Nature Structural Biology 5:765,1998). Whereas Botox A selectively cleaves the Gln187-Arg203 bond near the C-terminus of SNAP-25, the other botulinus neurotoxins each selectively cleave a different peptide bond within one of the three target proteins which comprise the SNARE complex.

A repeating motif exists within the sequences of all three of the proteins of the SNARE complex which, when introduced in the form of synthetic peptides of about ten residues in length, inhibits the actions of botulinus toxins on the SNARE complex (Rossetto et al, Nature 372: 416, 1994). For example one version of the repeating motif present in VAMP, designated V2 and having the sequence 62ELDDRADALQ71, blocks the neurotoxic actions of Botox A and B when the peptide is injected into cultured Aplysia neurons. The significance of the repeating SNARE motif, which appears twice on each of the four threads of the SNARE helix bundle upstream of the cleavage sites, is not well understood. Presumably it acts as a recognition site for binding of some other biomolecule(s), and may also be used by Botox as a binding-recognition element. In agreement with this, cross recognition of the target proteins by the various toxins occurs: Botox A inhibits VAMP proteolysis by Botox B, and Botox B and tetanus inhibit the cleavage of SNAP-25 by Botox A. Moreover serum albumin, which contains SNARE motifs within helical regions of its secondary structure (183DELRD187 and 255DDRAD259), inhibits the cleavage of synthetic substrate by Botox A.

Thus although the mechanism of action of V2 (and other variants of the SNARE motif) is not proven, it could involve binding to a complementary recognition site on Botox resulting in inhibition of productive binding of Botox to the SNARE complex. Interestingly, possible complementary SNARE motifs (592KKVNK596 and 701KRNEK705 in the heavy chain, and 335KLKFDK340 and 359KVLNRK364 in the light chain) exist in Botox A.

STRATEGY

Several possible strategies for inhibiting Botox exist:

- Block the transmembrane domain of Botox and prevent cell entry, e.g. complementary peptides, antibodies;
- Block the proteolytic site of Botox, e.g. substrate inhibitors, selective chelation of zinc;
- Block the interaction of Botox with binding-recognition motif, e.g. mimics of SNARE motif, which bind to complementary site(s) on Botox.

The first strategy has potential application prior to exposure to toxin (e.g., immunization), as well as for the deactivation of toxin in body fluids after exposure. The latter strategies, on the other hand, have the potential for providing total body treatment after toxin exposure - assuming that the therapeutic agent is able to cross cell membranes and inactivate toxin which has already entered the cell. Whereas agents based on active site inhibitors will have to be tailored to individual variants of Botox, therapies based on the SNARE motif have the potential to treat poisoning by all forms of Botox as well as tetanus.

Active sites of zinc proteases

The most widely studied of all the zinc proteases is carboxypeptidase A (CPA), and information on the mechanism of action of this enzyme serves a useful basis for understanding other related zinc enzymes such as Botox.

Detailed X-ray studies on CPA have illustrated that the tetradentate zinc atom sequesters a single water molecule and is tethered by coordination to the imidazole groups of His-69 and His-196 as well as to the carboxylate of Glu-270. CPA undergoes a conformational change on binding of the substrate Gly-Tyr, in which the guanidinium group of Arg-145 moves 2A to form a salt bridge with the C-terminus of the substrate, the carboxyl group of Glu-270 moves 2A away from the zinc atom and forms a salt bridge with the amino group of the substrate (can only occur with dipeptide), and the phenolic group of Tyr-248 moves 12A to within 3A of the scissile bond (Reeke et al. PNAS 57, 2220, 1967). Chemical modification studies have suggested that Tyr-248 is essential for the peptidase activity, but not the esterase activity, of CPA. A catalytic mechanism has been proposed in which the susceptible carbonyl oxygen of the substrate coordinates to zinc and the Glu-270 carboxylate attacks the carbonyl carbon to form a mixed anhydride intermediate, which is subsequently hydrolyzed by a base catalyzed mechanism involving the nascent water molecule (Zn-OH).

The best inhibitors of CPA are generally compounds that coordinate with the active site zinc and either sequester the zinc atom away from the enzyme or prevent access of the substrate to the catalytic center. These include thiols, amines/imines, flavonoids, and a range of anionic groups including carboxylates, phosphonates, etc. The most potent inhibitors will be those having an exact fit to the active site of the enzyme which is accompanied by strong attractive forces resulting in high affinity binding not only to the zinc atom but also to the enzyme's peptidic groups.

Due to its preference for basic amino acid residues, the enzyme most like Botox A is carboxypeptidase B. CPB differs from CPA in that it contains three disulfides (CPA has only one) and the coordination of zinc involves a free Cys thiol. Exchange of zinc for cadmium in CPB results in only esterase activity (no peptidase activity). Replacement of Asp-253 (which normally binds the basic sidechain of the substrate) with Lys gave a reversed-polarity mutant of human CPB which hydrolyzed hippuryl-L-glutamic acid (Edge et al, Protein Eng. 11, 1229, 1998).

The best inhibitors of CPA are benzyl- or alkyl-succinic acids and, assuming some parity between CPA and CPB, likely inhibitors of CPB would be aminoalkyl- or guanidinoalkyl-succinic acids. For reasons of specificity, it follows that those considerations which apply to CPB will likely also apply to Botox A. Accordingly, peptide libraries directed towards the active site of Botox A should be rich in basic and acidic moieties. Interestingly, acidic residues are also a characteristic of the SNARE motif, so that peptide libraries composed of these residues should interact with both the catalytic site and the SNARE motif site and act as dual inhibitors.

Regardless of mechanism, one approach to producing improved inhibitors of Botox is to identify the structural elements of V2 (and other SNARE motifs) that make it an effective inhibitor. Then reconstruct these components into a smaller and preferably nonpeptidic molecule, which would be able to traverse membranes, thereby providing not only access to the inside of cells, but also the potential for oral activity. A streamlined and efficient approach to this goal is to create a library of small semimimetic peptides containing the essential elements of the repeating motif in the SNARE complex, identify the most active peptide in the mixture by iterative deconvolution of the library, and then restructure the best semimimetic peptide identified into a fully fledged nonpeptide mimetic using computer molecular modeling techniques.

The important structural feature of the SNARE motif is comprised of an amino acid sequence made up of residues:

A-A-x-x-A-x-x

Where A = acidic residue and x = nonpolar or polar residue.

Due to the helical arrangement of these groups within the secondary structure of the SNARE proteins from which they are derived, the end result is a cluster of three neighboring negative charges juxtaposed by a nonpolar moiety. In other words, the required motif can be envisaged as three negatively charged groups, mounted in close proximity on a hydrophobic template. The simplest representation of this that comes to mind, is a benzene ring with three carboxymethyl groups attached at the 1, 3 and 5 positions. Other variations on this theme can also be easily

envisaged, and a number of commercially available compounds that fit this general scheme are being investigated for inhibitory activity in our laboratory, together with several peptide libraries which have been designed and synthesized with these considerations in mind.

DESIGN AND SYNTHESIS OF PEPTIDE LIBRARIES

One library has been synthesized which is designed to mimic the SNARE motif:

SN-1 = Ac -X1-X2-LINKER-X3-X4-NH2,

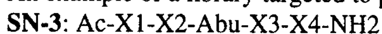
Where X1, X2, X3 & X4 are mixtures of Asp, Glu, Gln and Arg, and the LINKER group is 4-aminobutyric acid

The rationale for the design of the peptide minilibrary SN-1 is based on the preponderance of acidic residues in SNARE motifs, together with the occurrence of other residues in SNARE motifs which can provide solubility properties to the libraries – hence the inclusion of Arg and Gln in SN-1. The multiple negative charges represented by the Asp and Glu residues, when mounted around the flexible linker group 4-aminobutyric acid (Abu), should engender structural and conformational variations on the SNARE motif which will inhibit Botox.

Coincidentally, the Gln and Arg residues in SN-1 are also representative of the scissile bond (Q-R) in the Botox A substrate, so that the peptide minilibrary SN-1 doubles in a rudimentary way as a substrate mimic. This parallels the classic approach to inhibitor discovery, which is usually done by minor modification of substrate structure leading to compounds that bind to the active site but are not cleaved by the enzyme. Accordingly, incorporating residues of the scissile bond (Gln-Arg) into peptide libraries such as SN-1 should provide structural and conformational characteristics that might result in substrate-based inhibitors. Clearly SN-1 contains the potential for containing inhibitors based not only on the SNARE motif but also on substrate structure.

Another consideration for the design of peptide libraries with inhibitory potency is the inclusion of residues which would be expected to coordinate to the active site zinc atom of Botox, namely Cys and His as well as acidic residues. The approach here is to create a peptide library containing an array of zinc binding elements in a variety of different formats, wherein there should be individual peptides which recognize not only the zinc atom but also unique aspects of the active site of Botox A in which the zinc atom resides. The intent is to produce zinc targeted inhibitors that show selectivity for Botox over other zinc metalloenzymes.

An example of a library targeted to produce active site zinc inhibitors is:-



SN-3: Ac-X1-X2-Abu-X3-X4-NH2

where Ac = acetyl, Abu = 4-aminobutyric acid, and X1, X2,. X3, and X4 are mixtures of the amino acids Asp, Glu, His and Cys.

This library contains both active site zinc directed probes *and* variations on the SNARE motif within the same basis set (library) of peptides.

CONFORMATIONAL PROPERTIES OF TARGET BINDING SITES

Before intricate design work on inhibitors of Botox can be undertaken, details of the three-dimensional conformation of the target sequence must be elucidated. In the case of Botox A, the substrate SNAP-25 is believed to form part of a four-helix protein bundle that is anchored into the presynaptic membrane. Actually the cleavage site targeted by Botox A is near the C-terminus of SNAP-25, which is far removed from the central lipid anchor region of SNAP-25. Similarly the amino acid sequences representing the four SNARE motifs of SNAP-25 (S1, S2, S3 and S4) reside in the central part of the SNARE complex away from the lipid anchor domain found at one end of the tubular bundle. Accordingly, it is not clear if the environment surrounding the sequences representing not only the cleavage site but also the SNARE motifs is primarily polar (aqueous) or nonpolar (membrane lipid) in character.

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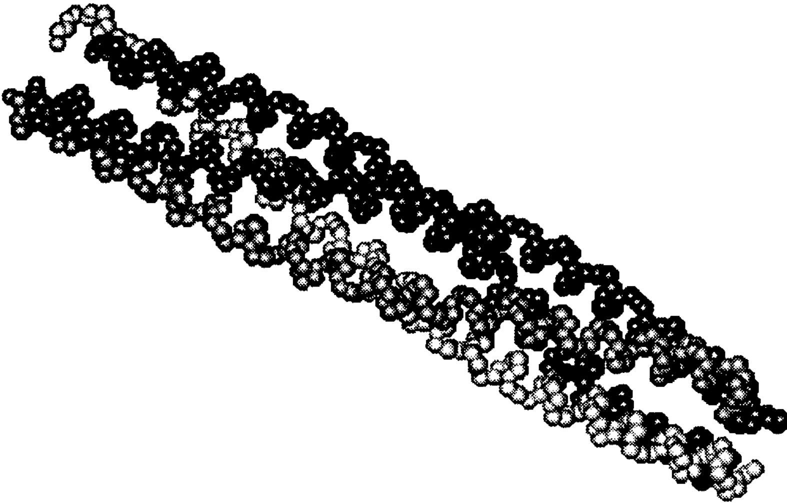
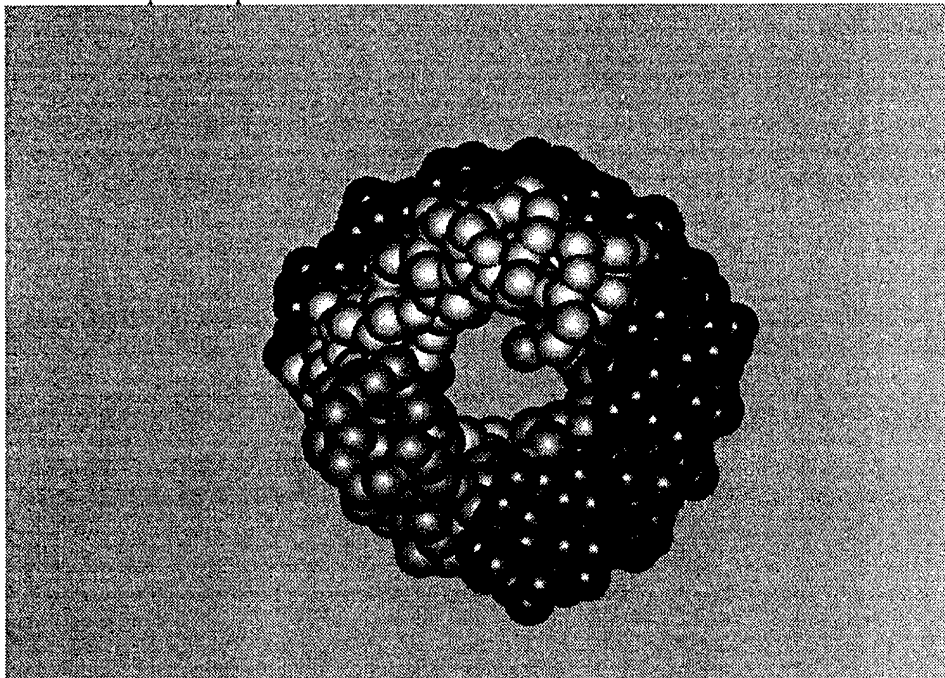
7. Edge et al Protein Eng. 11: 1229 (1998).

FIGURES AND TABLES

SNARE Complex: Lateral



SNARE Complex: Top View



Details of SNARE complex, showing the substrate, V2 and S3

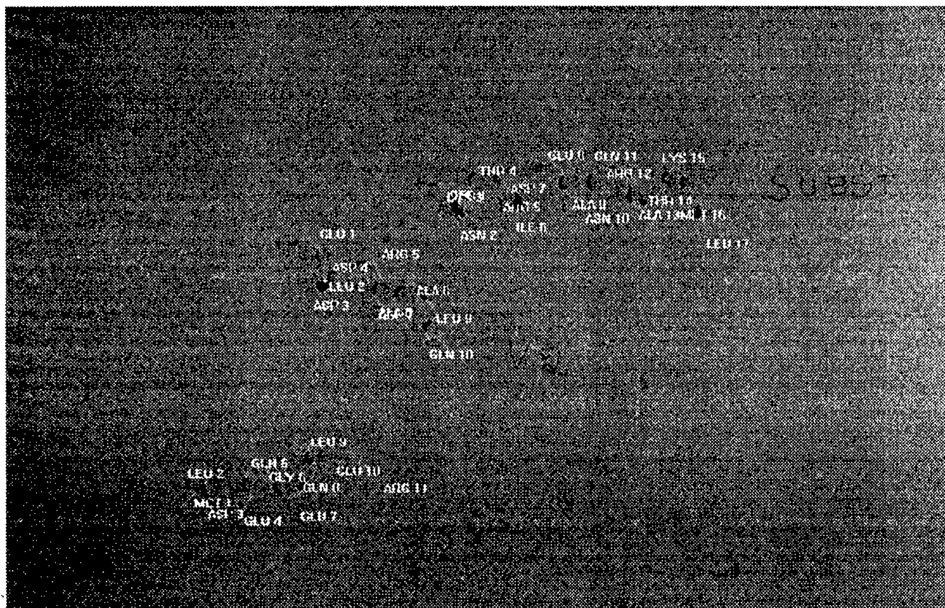


Table 1. Compounds found to inhibit the Botox A mediated cleavage.

Inhibitor	Concentration (mM)	% Inhibition*	-SH Concentration (mM)
Dithiothreitol	1	26	2
DMPS[‡]	0.1	72	0.2
Captopril	1	76	1
Lysinopril	5	7	0
Enalapril	5	40	0
V2 (ELDDRADALQ)	5	40	0
Glu-Glu-Glu	5	22	0
Glycyrrizic acid	5	55	0
Library SN-1	5 (20 _M)[†]	16	0
Library SN-3	0.5 (2 _M)[†]	51	0.125

9. HEALTH CANADA'S RESPONSE TO BIOLOGICAL / CHEMICAL TERRORISM

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CANADA'S NATIONAL COUNTER-TERRORISM PLAN (Interim, July 1998)

1. In the event of a Nuclear, Biological or Chemical terrorist threat, Health Canada (HC) develops scientific and human health assessments and provides certain services in the event of a major emergency. HC also assists in the identification, investigation, prevention and control of human disease, as well as the monitoring and investigation of infectious diseases.

2. A special committee meets to assess the nature, credibility and feasibility of any NBC threat, assesses the short and long term consequences of its execution and recommends mitigating and preventive measures, advising on recovery measures to senior bureaucrats.

3. Also available are: an NBC Response Team, a Hostage Rescue Unit, and various Operations Centers.

HEALTH CANADA ROLES (provisional)

- National guidelines/standards,
- Training/awareness,
- Risk communication,
- Expert knowledge regarding public health issues & appropriate health control measures,
- A research agenda,
- Drug and medical equipment stockpiling,
- Epidemiological support:
  surveillance (including field work as needed)
  requirements for post-exposure prophylaxis - exposed vs. unexposed people;
- Diagnostic and laboratory investigative support,
- Partnerships,
- Medical countermeasures such as drug regulation and the Special Access Program (SAP) - which gives authority to individual doctors for the use of unlicensed products in special circumstances.

THE ADVISORY GROUP ON NBC INCIDENTS (AGNBC):

The AGNBC is a small group of professionals who advise Health Canada and our Emergency Preparedness Organization on the health implications of NBC agents.
The AGNBC's tasks are to:

Identify those agents most likely to be involved in emergency situations;
draw up clinical treatment protocols;
determine what stocks are needed to provide a ready availability of therapeutic Agents in the event of an NBC emergency;
liaise with the SAP program to ensure that adequate supplies of drugs and other therapies are available when the need arises;
provide advice on specific measures to protect field health workers in NBC situations and on the most appropriate care of those affected by such an attack;
develop strategies for the use of collected information (to be stored in a database);
develop a network of communications; and
assist in the consequence management of the results of such an attack

Current AGNBC members include physicians and pharmacists who are experts in the use of therapeutic products, adverse drug reactions, narcotics and medical devices; a member from the drug Special Access Program; and a liaison member who is in contact with inspectors in the regions/provinces, and who check for drug tampering & recalls for example. Other persons with specific areas of expertise may be called upon for advice as needed. In a CB emergency, AGNBC members are alerted by an operations center that is manned on a 24-hour basis. The Chairperson is a member of a larger interdepartmental committee that deals with all aspects of terrorism and NBC incidents.

THE NATIONAL MEDICAL STOCKPILE

Under the control of Health Canada's Emergency Services Division, the National Medical Stockpile was initially put in place in the early 1950s to support Canada's recovery after a nuclear attack. It has been modernized and now includes:

200-bed Emergency Hospitals for short term, emergency care;

- Advanced Treatment Centers (ATCs) that support field triage, early medical stabilization and transport of up to 500 casualties;
- Casualty Collection units: each one has initial supplies for the immediate collection, first aid and movement of up to 500 patients;
- Mini-clinics to supplement existing medical care facilities in emergency situations that overwhelm the local facilities;
- Mobile Feeding Units that are adaptable for use in any disasters;
- Trauma kits: Multi-component backpacks that support patient staging areas, mini-clinics, ATCs, etc. These include:
  - Basic Life Support haversack with first aid equipment & supplies
  - Advanced Life Support haversacks (3) with intubation, medical, IV supplies;
- Reception Center Kits: Registration/inquiry material and supplies for set up and operation of reception centers and congregate lodging; and

other equipment as needed.

Health Canada holds a limited stock of selected pharmaceuticals in support of the above assets and has standing arrangements with major pharmaceutical producers to store and rotate additional pharmaceutical supplies.

THE CANADIAN LABORATORY CENTER FOR DISEASE CONTROL (LCDC)

The LCDC provides the overall lead and the initial contact point within HC in the field of CB-CT. LCDC has laboratory diagnostic and biosafety services to aid in the positive identification of agents, and also has expertise in epidemiological and population surveillance, the diagnosis of patients, plus the public health aspects of bioterrorism (BT).

PARTNERSHIPS

HC has or is prepared to make co-operative arrangements with emergency health and social service agencies in all Canadian provinces and territories to provide needed support, either through the recruitment and deployment of treatment specialists into an affected area, or through the reception and temporary care of evacuated victims outside the affected province/territory, if needed. A two-day seminar was recently held with provincial representatives and those of the major Canadian cities to improve communications and to consolidate federal, provincial and local/municipal knowledge and plans.

HC has arrangements with volunteer Non-governmental Organizations (NGOs) whereby they will provide or assist in establishing and operating a victim/evacuee registration and inquiry service, as well as provide other assistance where needed. NGOs will also co-ordinate social service resources mobilized by themselves, international volunteers, organizations or appeals.

Other partnerships have been/are being finalized within the government, with local/municipal health departments, various advisory bodies and international groups.

CO-ORDINATION - The various initiatives and partnerships mentioned above are being brought together and some are being further developed by an Internal Working Group on instruction from the Federal Deputy Minister of Health.

THERAPEUTIC PRODUCT SHORTAGES

A 'shortage' is any situation where there is a potential that enough product will not be available to meet demand, thus resulting in patients being untreated. The special nature of the starting materials for therapeutic products and the variability in manufacturing processes means that the possibility of therapeutic drug shortages is a constant threat.

Roles & Responsibilities of Stakeholders: A manufacturer is not required by law to provide an uninterrupted supply of their product or to regularly report on supplies on hand. It is, however, in the best interests of stakeholders to work with the regulating authority by providing information and replenishing supplies as soon as possible. We should establish a shared responsibility for shortage management with manufacturers of critical products, their associations and other health associations. Associations could be enlisted to educate their members on Canadian management concerns so that their members could be sensitized to such concerns and be able to facilitate specific future shortage management activities as they occur.

The unique situations that will arise will require a flexible approach to overcome the problems that are sure to occur. The goals of such shortage management activity are illustrated in table 2.

STEPS NEEDED TO ANTICIPATE AND ALLEVIATE THE IMPACT OF SUCH SHORTAGES

1. Issue Identification - a clear identification of the problem aimed at limiting the expenditure of resources and determining whether a true product shortage is imminent. The factors most likely to cause a shortage are:
    - a single source of raw material (lack of alternative supplies),
    - a single manufacturing site,
    - rare raw materials,
    - potential for pathogens,
    - country of origin,
    - products with short expiry dates, and
    - an essential product list.

Three factors that must be documented are:
- the type of product (e.g. whole blood, vaccines, etc),
- the cause of the potential shortage (e.g. increased disease incidence, discovery of a new pathogen, insufficient raw material, lack of manufacturing capacity, distribution problems, industrial action), and
- names and addresses of manufacturers.

A list of critical drugs, based on the impact a shortage would have, should be drawn up. There are two criteria for designation of a drug as critical:
- it must be an essential drug ('essential to the basic health needs of the majority of the population' - World Health Organization),
- it must be a medical necessity, i.e. it will prevent or control the occurrence of an epidemic, is life-saving or a life-preserving drug for which there are no alternative products or intravenous fluids available, nor will a non-drug treatment suffice.

2. Assessment - analyze the cause and the impact of the issue. It is crucial that manufacturers be able to provide information on their stocks. Factors to be considered are:
    - the event:
        - probability of occurrence, the likely severity,
    - costs involved (also of the assessment),
    - likely duration of the shortage,
    - medical necessity (cause of the shortage, number and type of products affected by any one cause).
    - the absence/presence of an acceptable, approved product,
    - the geographical extent of the shortage.

3. Risk Management Tools & Options. These are used in anticipatory risk management and the management of 'at hand' specific shortages.

Prevention:
- international harmonization,
- anticipatory hazard evaluation,
- acceptable alternate supply plans,
- invest sufficient resources to monitor supply levels and detect potential shortages early on.

At Hand Situations:
- assist the manufacturer to correct the problem that led to the shortage; replace from another company or import from another country,
- preferential release of lots awaiting approval, expedited (priority) review of a product,
- the Special Access Program,
- discretionary enforcement of safety requirements,
- extension of expiry dates,
- special labeling, etc.
- Manufacturers should have prior plans for correcting shortage situations.

4. Decision. Who is to review the options, decide on a strategy and make the decision as to what actions to take? (This should be done in consultation with the stakeholders if time allows). The goal of the strategy is to reduce the risk to a level as low as reasonably possible. Consider benefits and costs, also ethical, political, legal and cultural implications. Allocation of resources should be prioritized according to the critical product list. It is better to emphasize the public health obligations of a company than to try to enforce compliance with regulations. Document all factors considered in your deliberations and decisions.

5. Implementation. Contingency plans and an action plan with target dates should be developed (who does what) and responsibility for the implementation of the strategy should be assigned

Performance measurements should assess whether or not information on the level of supplies was readily available, whether or not shortages actually occurred and if so, whether they were detected in a timely manner. They should also measure the time taken to respond to them, whether or not the original problem had been corrected, assessment of the costs incurred, and whether or not the strategy was appropriate, effective and acceptable to the stakeholders. The public health impact of the shortage and the strategy should be ascertained (# cases of illness averted, # cases of illness untreated, changes in incidence of a particular disease).

6. Monitoring and Documentation of each step in the process should take place.

7. Consultation & Communication. Decisions must be evidence-based and be acceptable to the stakeholders. Communication should be a two-way process involving consultation, which leads to greater compliance.

RECOMMENDATIONS FOR IMPLEMENTATION OF THIS PROCESS

1. A communication plan should be developed;
2. A team should be established to review and prioritize products, identify which anticipatory management actions are needed. Evaluate newly approved products for the list;
3. A Health Hazard Evaluation form should be developed and used;
4. Your Therapeutic Agency should undertake a continuing scan of the product supply;
5. Alternate supply plans should be made and the financial implications sorted out;
6. Countries should plan to become self-sufficient.

CONSEQUENCE MANAGEMENT

The government-led coordination and implementation of measures intended to mitigate the damage, loss, hardship and suffering caused by acts of terrorism, including measures designed to restore essential government services, to protect public health and safety, and to provide emergency relief to affected governments, businesses and populations.

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FIGURES AND TABLES

Table 1. Total # of Cases, Deaths, Hospitalization days and Cost after anthrax or botulinum toxin attack, if 100,000 of the population are exposed - a projection based on a CDC model using Canadian data.

The Threat:	ANTHRAX	BOTULISM
Total Cases	50000	50000
Total Deaths	32875	30000
Total Days of Hospitalization	332500	4275000
Total Cost	$6.5 billion	$8.6 billion

Table 2. Goals of Shortage Management Activity

1. To prevent any therapeutic product shortage from occurring;
2. In the event of a shortage or potential shortage:

determine the actual/potential extent of the shortage,
develop and implement appropriate allocation strategies for remaining supplies,
locate and deliver interim supplies or alternative products and/or a renewed supply,
determine and review the circumstances which led to or could have led to the shortage,
develop a communication plan,
develop and implement new or revised procedures to address or prevent the re-occurrence of those or similar circumstances.

10. ACTON'S NEW CB MOULDED GLOVE: "GLOVES THAT FIT AND DO NOT TEAR, A NOVEL IDEA"

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ABSTRACT

Acton International and DRES (Defence Research Establishment Suffield) are finalizing the development of a new Chemical and Biological (CB) Moulded Glove. The majority of CB (NBC) gloves today are made using a dip-coating process. This process is time and labor intensive and produces gloves with many shortcomings (i.e., high cost, poor durability, low dexterity, limited tactility and poor physical properties, especially poor tear strength). The Dip-coating process also uses large quantities of solvents that pose fire and environmental risks. Acton's new CB Moulded Glove employs novel moulding processes enabling the use of innovative polymer formulations with improved physical properties. The glove design is unique in that it was developed as a sizeable computer aided design (CAD) model that incorporates over 40 hand dimensions from an anthropometric database of over 550 soldiers. This design also addresses critical ease considerations identified in glove fit and dexterity trials carried out by the Canadian Department of National Defence.

The result is a new glove with far better fit, tear resistance and physical properties than comparable dipped gloves. The novel moulding technologies enable Acton to mould a glove thin enough to be used as a standard CB (NBC) Glove. Key design features of the new CB glove include: a design that is based on actual hand dimensions and proportions; snug fitting fingertips for enhanced tactility and dexterity (e.g., keyboard use, ammunition handling); corrugations on the main finger joints for better mobility; a textured finish on the fingertips for improved grip; and optimized polymer formulation for increased durability.

INTRODUCTION

In chemical and biological (CB) threat environment, dipped polymer gloves have been an important part of individual protective equipment. Depending on the degree of dexterity required to complete a given task, two types of gloves are available to the Canadian Forces. A 25 mils (0.622 mm) lightweight unsupported glove is used for fine manipulative tasks requiring good dexterity, while a 65-mils (1.5 mm) supported general purpose glove is used for all other tasks. Both gloves are made of butyl/chloroprene polymers and are worn without glove liners. A number of deficiencies which have been identified with dipped polymer gloves are inadequate resistance to petroleum, oils and lubricants (POL`s), poor physical properties (especially poor tear strength), limited manual dexterity, accumulation of hand perspiration and high production costs. To address these deficiencies the Defence Research Establishment Suffield (DRES) has been conducting research in a number of areas including moulding technologies, new polymer formulations, super absorbent layers, liner materials, selectively permeable CB gloves, and anthropometric requirements to optimise manual performance of gloved hands. Under a contractual arrangement with DRES, Kinection conducted all human factors studies and led the effort on integrating anthropometry into a CAD model to create an industrial design.

In a collaborative effort, DRES and Acton International have combined their expertise to finalise the development on the polymer glove. The new CB glove is being produced using a moulding process instead of the dip-coating process traditionally used for CB glove production. DRES has provided Acton, through a Licensing Agreement, an industrial design for a glove generated on a sizeable computer aided design (CAD) model, extensive sizing data based on military population hand anthropometry, polymer and liner expertise. Acton has contributed its moulding and formulation expertise to this project.

BENEFITS OF MOULDING PROCESS FOR CB GLOVE PRODUCTION

During the mid 1980's, the glove research program was directed at investigating new technologies for producing CB gloves. The dip-coated butyl latex glove provides protection against CW agents, but lacks the durability required for military operations. It has poor resistance to many of the solvents, fuels, lubricants, decontaminants and other common fluids used by the military. The dipping process is time and labour intensive, can use large quantities of solvents that present fire and environmental concerns during glove manufacture and it has high production costs. Dip-coating, using latices or solvent solutions, limits the use of intricate design considerations. Latices cannot be compounded to give the required properties and solvents.

The new CB glove is produced using a moulding process instead of the dip coating process traditionally used for CB gloves. Moulding was explored during a feasibility study to investigate alternate methods of producing gloves. A prototype glove shape was machined and gloves were successfully moulded on a small scale in a research lab. The compression molding process was chosen over other processes used for glove manufacturing for several reasons. Gloves fabricated from calendered sheets require sealing of seams and have limited design capabilities. Injection and transfer moulding require more expensive complex molds and machinery, and have complicated mold flow patterns which can lead to precuring of the rubber and exacerbate mold core deflection. The selection of a moulding process offers a greater selection in materials than a dipping process.

Throughout the development phase, a number of new rubber compounds were formulated. The specific elastomers and compounding ingredients used were selected through an extensive investigation of commercially available materials. The challenge was to develop a formulation for a polymer material that offers the best compromises and balance between compatibility of component materials, broad-spectrum chemical resistance, good physical properties and acceptable processing characteristics. The results for important physical properties of materials for dipped and moulded gloves for one of the candidate materials were normalized for comparison and differences are shown in Figure 1. Durability, as represented by tear strength and puncture resistance was significantly improved. The tear strength of some of the new formulations is twice as strong as the materials from dipped CB gloves. Tensile strength was also improved even after materials were exposed to various field POLs. While elongation at break decreased it should be noted that this property influences ease of demolding and flexibility of the glove and the level achieved in the new materials were still above the minimum desired range of 600%.

GLOVE DESIGN, SPECIAL FEATURES AND SIZING SYSTEM

The unique design for the moulded CB glove was developed by DRES in the form of a sizeable CAD model. The design was developed through careful consideration of many factors including hand anthropometry, ease requirements, user fit and comfort. Computer aided design, anthropometric studies, dexterity tests and user-acceptability surveys were used to develop the shape and sizes of the glove form. The approach adopted was to develop and optimise the glove design in only one size and then to implement a sizing system to a computer generated CAD glove model to generate the necessary number of sizes for a user population. The use of a CAD model enabled the integration of human anthropometry and manufacturing process considerations while maintaining a practical mould design. It also gave a direct link to computer aided machining for mold fabrication (Figure 2).

The new CB glove design is the end result of a series of dexterity and fit evaluations on successive CB glove prototypes (1,2,3,4). The glove was designed as a relatively thin (22 mil ± 0.3 mil) multipurpose CB protective glove. It was designed to provide a snug fit for good dexterity in fine manipulative tasks. The CB glove was designed according to the sizes and shapes of "real" hands; i.e., the CAD glove models incorporate over 40 hand dimensions from a current, comprehensive hand dimension database comprised of 53 direct measurements on 358 male and 178 female soldiers (5,6) (Figure 3). The design dimensions for the new CB glove were developed with donning in mind, and clearances for clothing, mobility and dexterity have been addressed. Positive ease allowances in girth have been incorporated at the cuff, the wrist, the palm, and the area of the thenar prominence (i.e., the large muscle at the base of the thumb) (6). A slight negative ease has been incorporated into the finger lengths of the glove for enhanced manipulative task performance (7). In other words, when compared to anthropometric parameters, the length in the glove fingers is actually slightly shorter.

Several generations of glove models led to the final glove design. A rapid prototyping method called stereolithography (SLA) was used throughout the development process to directly fabricate glove forms from the CAD files. SLA forms were extremely useful to verify modifications made to the glove shape and the forms were also used to dip gloves for dexterity and fit evaluations (Figure 4). The CB glove design is flat and symmetrical. While both flat and curved glove shapes were studied, the flat glove configuration was chosen to provide a relatively uncomplicated mould design and ease in fabrication. Unlike a natural curved hand glove design, the flat glove has no designated right or left hand glove. This aspect of the design provides obvious donning and replacement benefits, in addition to reducing the production cost of moulds. To enhance hand mobility, corrugations were placed strategically at the following critical joint locations: the thumb crotch, the palm at the metacarpal heads, and the proximal interphalangeal joints of the index and middle fingers. The corrugations in the glove facilitate flexion of the hand at these joints and provide a degree of adjustability in the length fit of the index finger, middle finger and palm (4). Further glove features include surface texturing on the fingers for improved grip.

The new CB glove design uses an integrated (i.e., combined male and female) seven size system. The seven-size system was developed by DRES using established anthropometric sizing techniques in combination with in-service

CB glove and prototype CB glove fit assessment data (4,6). The sizing system is designed to accommodate hand sizes ranging from 155 mm in hand length and 168 mm in palm girth to 211 mm in hand length and 239 mm in palm girth (6). For all but the smallest size, this sizing system provides two palm girth size options for each hand length size interval. Two of the seven sizes are predominantly for females, two of the sizes are mixed gender, and the three remaining sizes are predominantly for males.

The design dimensions for the new CB glove were developed such that the glove could accommodate a thin glove liner. While the use of a glove liner increases the overall thickness of a glove and therefore has dexterity and tactility implications, it can improve the "feel" of the glove. Liners provide a natural fibre layer between the skin and the polymer and can absorb some of the sweat/moisture that accumulates with the use of an impermeable glove. During the prototype development stage, more than 10 material combinations were knit into gloves using both a plating technique and a speckled technique (8). Plating technique knits two different yarns simultaneously while controlling the position of yarns on the inner and outer most surfaces. Speckled technique knits the two yarns simultaneously and allows the yarns to twist throughout the glove. For CB moulded glove liners, the recommendation for the best combination of yarns are Coolmax™/Lycra and viscose, knit in a plated structure. By adding Lycra, the liner provides a snug fit and reduces excess bulk inside the polymer glove. When the plating technique is used to knit the yarns, the gloves acquire a slightly better wicking ability. The glove liner was assessed during dexterity trials and demonstrated excellent fit and user acceptability with minimum decrement to hand manipulation (4,7).

ACTON'S NEW CB MOULDED GLOVE: THE FINISHED PRODUCT

The result of the partnership between Acton and DRES is a new CB glove with far better fit, tear resistance and physical properties than comparable dipped CB gloves. The novel moulding technologies employed by Acton enable a glove to be moulded that is thin enough to be used as a standard CB (NBC) glove. The moulded CB glove features snug fitting fingertips for enhanced dexterity (e.g., keyboard use, ammunition handling), a textured finish on the fingers for improved grip, corrugations on the palm and main finger joints for enhanced mobility, a design that is based on actual hand dimensions and proportions, a sizing system based on analysis of current anthropometric data, and an optimised polymer formulation.

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KEYWORDS
CB protective glove, moulded glove, glove design, hand anthropometry, glove sizing, ease, corrugation

FIGURES AND TABLES

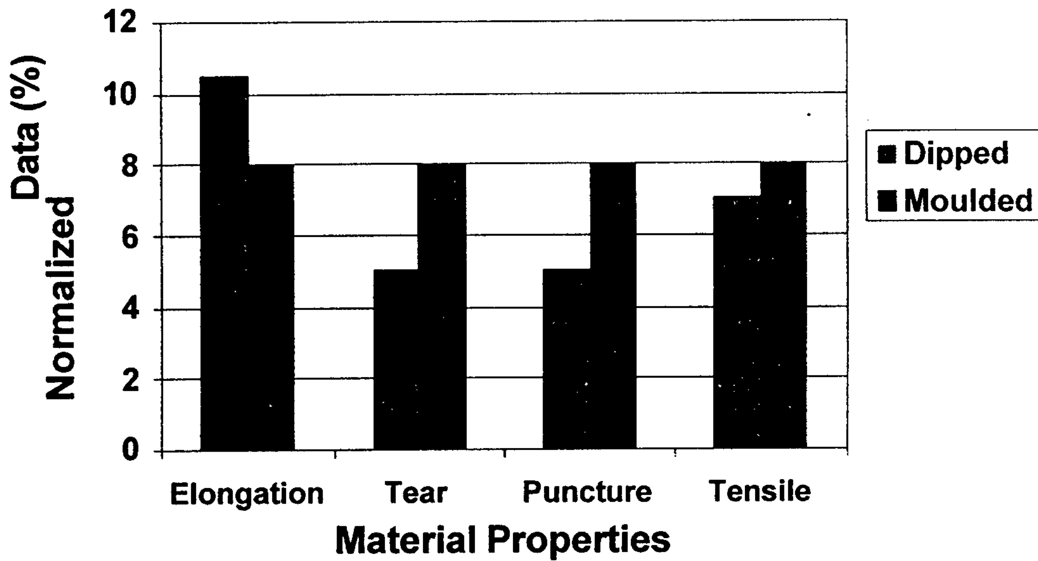

Figure 1. Comparison of normalised data for physical properties of dipped and moulded glove materials.

Figure 2. Mould for new CB moulded glove machined from computer generated CAD glove files.

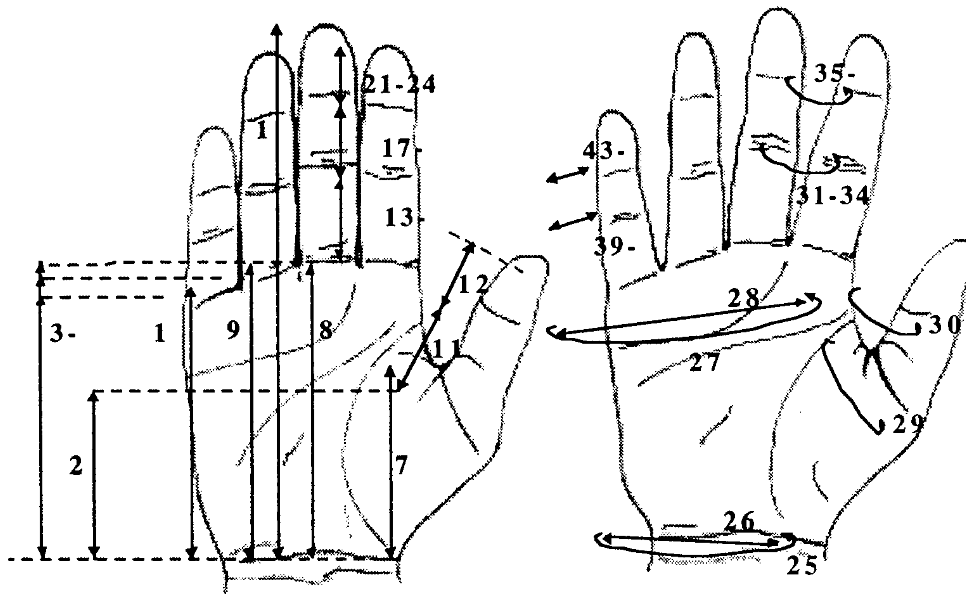

Figure 3. Hand dimensions from anthropometric database used to develop the industrial glove design generated on a sizeable CAD model.

Figure 4. Rapid prototyping form of an early glove design used to dip prototype gloves for design verification.

11. AN ASSESSMENT OF TOXINS

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INTRODUCTION

Toxin weaponry is a new category of the chemical-biological agents spectrum and is especially a challenge to future chemical-biological medical treatment. Obviously, there is a need for assessing the future threat from toxin weapons and determining its development in the future. But many questions often arise regarding the assessment of toxins. Therefore, an overlook discussion will be made in the present paper.

DIVERSITY IN TOXINS

In scientific terms, toxins are biogenic nonreplicating toxic natural substances, which cover an immense range of compounds found in plant, animal and microbial organisms. It is increasingly realized that toxins are not needless natural products of organisms. On the contrary, they are primary products or secondary metabolites with specific functions in biology. Toxins have been shown to play a critical role in the behavior and ecological interaction of different kinds of organisms; the toxins have often likely evolved for specific biological functions, such as defense, offense, digestion etc. On the other hand, the isolation of natural toxins and the study of both the chemical knowledge and the biological information about their functions and specificities have provided a foundation for new drug and agrochemical research.

Diversity is an essential character of nature and a colorful display of life; it seems that toxins also display a distinguishing feature of diversity in many aspects as biogenic sources, chemical structure, functions, and mechanisms of action.

Biogenic diversity

It is the fundamental feature of diversity in toxins; thousands of poisonous substances are produced by a variety of living organisms from various phyla. These include a great quantity of species of living organisms, such as bacteria, fungi, plants, insect, reptile, amphibious animals, and numerous genera of marine organisms. All of these can produce a huge variety of compounds with very different toxic actions.

Chemical diversity

Toxins are a distinct class of active biomolecules in the nature, which span a wide variety of chemical structure types from extremely complex biomacromolecules to very simple organic and inorganic compounds (See Table 1). Furthermore, a number of noticeable structure types in natural toxins do not appear in synthetic molecules. It is obvious that the chemical diversity of natural toxins provide a rich source of lead structure for drug design or biotechnological application.

Diversity of action

Natural toxins act in many different ways on many different life systems and processes in the body. They usually specifically target important enzymes, cell membrane structures, ion-channels, receptors, ribosomal proteins, etc., to induce a vast array of lethal and non-lethal toxic effects. This characteristic diversity is also valuable because it provides new opportunities and selectivities for new drugs and bioactive substance development.

The diversity of natural toxins is very attractive to scientists of biology, chemistry, medicine, drug research and many approaches in life science; research in natural toxins became a new interesting inter-discipline subject - toxinology - in recent decades. In the meanwhile, it implies a risk threat that rapid development in natural toxins open up new opportunities for military purposes.

TOXIN AGENT IN/OUT TOXINS

When we talk about toxins in the chemical biological defense (CBD) arena, the word "toxin" is applied as an abbreviated form of the military term "toxin warfare agents" or "toxin weapon." But, the same word "toxin" in the scientific and CBD fields may have quite different messages. It is true some natural toxins should be listed in the spectrum of toxin warfare agents; however, toxin warfare agents may consist of other meaningful military toxic substances derived from natural toxins indirectly as well. In short, we can say toxin agents are in natural toxins and out of them.

Based on potent toxicity of natural toxins, there are great possibilities and promotive forces to select some of the most poisonous toxins as toxin weapons. Several hundreds of known toxins exhibit toxicity higher than classic nerve agents. It is well known that some toxins from bacteria or marine organisms, botulinum toxin, palytoxin,

maitotoxin are even 1,000,000 times more toxic than nerve agents. From one Data Bank of Poisons and Toxins, the data show that in the collection of more than 8000 toxic substances, including synthetic poisons and natural toxins with toxicity up to 10 mg/kg, all known most potent toxic substances, whose toxicity is higher than 0.1 μg/kg, can be classified as natural toxins (As shown in Table 2.) But it is also true that a series of factors other than toxicity may play a major role in developing natural toxins as toxin weapons. Not unexpectedly, up to now, only a very few natural toxins have been reported to be weaponized, stockpiled or used.

On the other hand, especially if one could look into the future, more and more opportunities for toxin weapons may result from indirect approaches to find new toxin agents by making use of the achievements in natural toxin researches. At the present time, it is an easy way to create valuable derivatives or modifiers of known toxins through chemical and biotechnological approaches to overcome their critical shortcoming. The derivative structures may be related to but different from original natural toxins. Furthermore, rapid advances in chemistry, molecular biology, biotechnology and drug design technology greatly facilitate the search for new drugs and other active substances with novel structure or scaffold. Based on the information of mechanism of action and target receptor structure of known natural toxins, it is possible to elaborate hitherto unknown types of toxic compounds by means of de novo design approach. These toxin-mimics and artificial toxins (a novel class of toxins), may have similar specific and potent actions to natural toxins, although their structural appearance may be different from the original toxins. Therefore when assessing the toxin threat, these new derivatives, modifiers, mimics and artificial toxins must be considered in addition to natural toxins.

Hence, the toxin warfare agents arise from natural toxins and toxiconological researches have inherent relationships with natural toxins, but the scope and construction will differ greatly from each other (As illustrated in Fig. 1).

ASSESSMENT FACTOR

Many assessments of toxins have discussed and organized toxins mainly on their toxicity basis. Some authors have pointed that a set of critical factors must be considered in assessment of toxins other than toxicity. And many primary factors may be contributed from chemical essence of toxins, so it is important to assess toxins from a chemical viewpoint.

In general, the primary assessment factors of toxins should consist of following set:

Toxicology Factors

This set of factors should include toxicity potency, intoxication route as inhalation, percutaneous etc., onset time and other related factors. Undoubtedly the principal route of intoxication of toxins in the case of military use is inhalation. It is important to note that although it seems unreasonable, and the mechanism is not clear, the experiment results show some toxins have higher potency by the inhalation route even than by injection. This is in large contrast with the toxicological property of known chemical warfare agents such as nerve agents (As shown in Table 3).

Application Factors

These are closely related to weaponizaton of toxins. Among the essential factors of this set are dissemination property and stability in different environmental conditions. The package technology may have an important effect on stability property.

Production Factors

Toxins can be obtained by biochemical processes, synthetic routes and biotechnological methods (i.e., fermentation, gene manipulation, etc.), but often in relatively small quantities. The key issues are degree of difficulty in technological requirements, cost and material availability of applicable methods. Meanwhile, it is noteworthy to note the dual-use property of manufacture process. In this case, it could facilitate to the last degree for production.

R&D Factors

At present, it seems no one toxin can be chosen as a "good" toxin agent. Each of the known toxins reveals some remarkable inadequacy in one or more aspects of the assessment factors mentioned above. However, our concern is not only for the present time, but also to have tools to evaluate toxins in the future. So when we consider the assessment issue of toxins as a dynamic task, then R&D factors become key in toxin assessment. In this group, we should pay more attention to the following factors:

1) Discoveries in novel structure types of toxins and related approaches in structure reconstruction.

2) Advances in new production technology as applicable technology in stereo-synthetic chemistry, new methods from biosynthesis pathway and new achievements in biotechnology.

3) Discovery of new target receptor systems of toxin, which could be used as template for novel series of chemical structures with high specificity.

ASSESSMENT OF TOXINS AT DIFFERENT LEVELS

In fact, when we talk about assessment of toxins, the question may involve some issues at different levels, and may be to get at divergent opinions or conclusions.

Firstly, at the category level, that means regarding toxin agents as a new field in chemical and biological agent spectrum. In this case, much of the information indicates an affirmative deduction, which classifies toxins as a real threat in addition to classic chemical and biological agents.

Secondly, there is a need to assess toxins according to their different types at class level. Several ways of classification can be applied to toxins. Because a series of primary assessment factors may be contributed from chemical essence of toxins, it is important to assess the toxins on chemical classification first, rather than on other basis.

According to *chemical structure type*, toxins could briefly be classified into the following classes:

1) Proteins: enzymes, bacterial toxins
2) Peptides: a variety of venoms from animals
3) High-stereo organic compounds such as palytoxin, maitotoxin etc. polyether toxins from marine organisms;
4) Organic compounds: numerous toxic substances from plants, fungi, and marine life.

To assess toxins at the class level, scientific discussion and information shows that the peptides and high-stereo organic compounds receive heavy attention for their high toxicity, have acceptable characteristic in application, and are potentially availability through chemical synthesis and genetic engineering in advance. More favorable feature may be derived from assessment of toxins on a gradual subclass basis.

Finally, to assess toxins at the individual level is helpful to chemical and biological defense work, but also it is a very complicated, even impossible task at the present time. No one toxin fits all the necessary requirements of a toxin agent; all have too many characteristics of uncertainness and immaturity relative to individual toxins; hence, even at the individual level, assessment of toxins is probably senseless or impossible. On the contrary, selecting an individual toxin as a protoype or representative substance of various toxin classes is a necessary step to make assessment of any case. Then, the discussion may lead to real conclusions and future prospective figures, which is a great necessity for chemical and biological defense work.

FUTURE TOXIN AGENT SPECTRUM

Of toxins that have been stockpiled or used, there is a small group consisting of botulinum toxin, diphtherin toxin, staphylococcal enterotoxin, ricin, saxitoxin and T-2 toxin. At most, we could also include dart poisons in this history. Their military significance and the intelligence of the information of their use can still be debated. Nevertheless, in the CBD and chemical and biological disarmament field, the scope is widened far beyond the list mentioned above. At least 400 toxins have been discussed in CWC or BWC related activities. The whole range of toxins from different sources such as bacteria, animals, fungi, plants and marine life organisms were investigated from the view of military risk assessment. Why? It is really these things that bring the attention to the future toxins spectrum. From our discussion on the assessment of toxins, there is yet no possibility to construct a clear future toxins spectrum today. Certainly, the future toxin spectrum is more complex, more variable and more unpredictable than known chemical or biological agents spectrum.

In summary, the goal of assessing toxins for chemical and biological defense requires the construction of a future toxin agent spectrum, but this is a dynamic and complex task.

KEYWORDS

Toxin, toxin agent, toxin spectrum

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FIGURES AND TABLES

Figure 1. Toxin agents in and out toxins

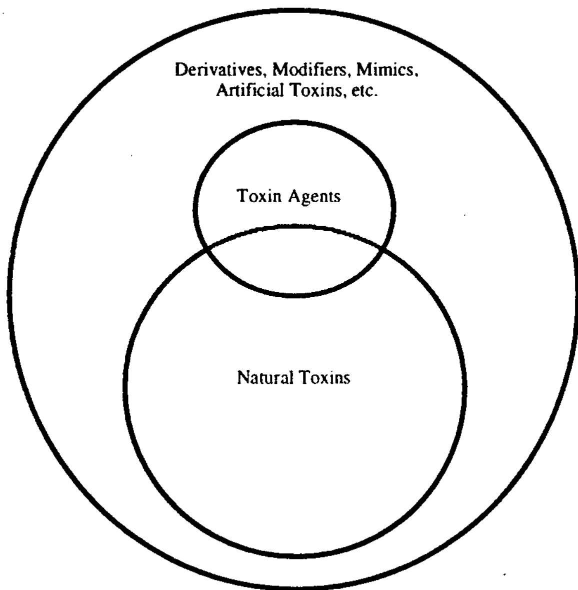


Figure 2A Maitotoxin

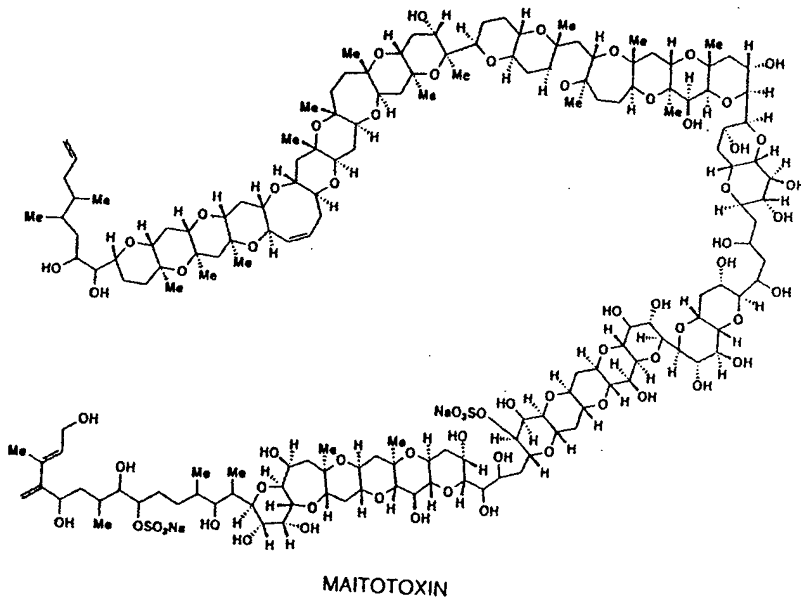
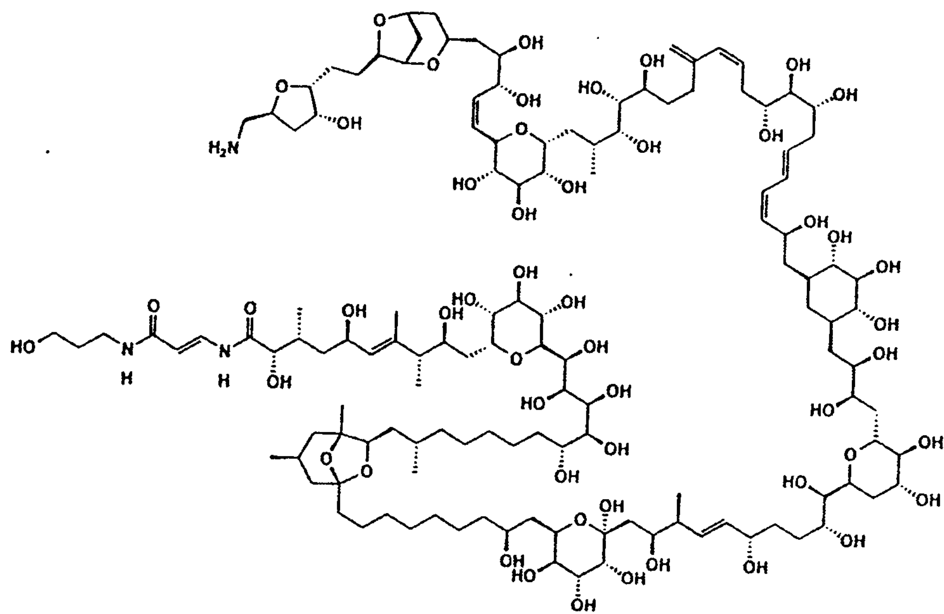


Figure 2B. Palytoxin



PALYTOXIN

Table 1. Chemical Diversity in Toxins

Plants		Target	Toxin
proteins	40	Seeds	Ricin,abrin
cyano-glycosides	30	Fruit kernel	Amygdalin
saponin	100	Widespread	Phytolacecatoxin
sesquiterpene	200	Compositae	Picrotoxin
diterpenoids	200	Resins	Grayanotoxins
alkaloids	2000	Angiosperms	Tubocurarine, aconitine

Table 2. Ratio of Toxin Toxicity to Poison

Toxicity ug/kg	Number of Toxins A	Number of Poisons B	Ratio Toxins/Poisons A/B %
< 10,000	1589	7295	21.6
<5000	1371	5149	26.6
<1000	889	2640	33.7
<500	677	1948	34.7
<100	310	910	34.1
<50	192	610	31.5
<10	96	196	49
<1	43	52	82
<0.5	34	39	87
<0.1	20	22	90.9
<0.01	13	13	100

Table 3. Inhalation Toxicity of STX and Sarin

Agent	LD_{50} µg/kg mouse i.v	LD_{50} µg/kg mouse i.h	LC_{50} µg.min/L mouse i.h	LC_{50}µg.min/L human (calc.)
STX	8	0	3	2
Sarin	42	100	200	70-100

12. THE EFFECT OF SOME REVERSIBLE CHOLINESTERASE INHIBITORS ON THE REACTIVATABILITY OF SOMAN-INHIBITED HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE IN VITRO

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INTRODUCTION

Organophosphorus (OP) agents can cause an irreversible inhibition of Acetylcholinesterase (AChE, EC 3.1.1.7) by phosphorylating or phosphonylating a serine hydroxyl at the active site of AChE, resulting an accumulation of acetylcholine in the effector organs. The causal antidotal therapy for this intoxication consists of anticholinergic drugs to counteract the accumulation of the AChE reactivators, which can restore the enzyme activity by removing the phosphonyl moiety. But due to the rapid dealkylation ("aging") of soman (1,2,2-trimethylpropylmethylphosphonofluoridate) inhibited AChE, soman phosphonylated AChE is resistant to most of the pyridinium oximes, such as 2-PAM.Cl, Toxogonin, TMB_4, which causes that the therapy of soman poisoning is notoriously difficult. The search for drugs that can effectively reactivate the soman-inhibited AChE remains an urgent task.

In the late 1970's, Harris et al. had reported that SAD-128 could obviously increase the reactivating ability of soman-inhibited AChE (1). From then on, other investigators also found that some other compounds, such as atropine, methantheline and several ammonium halides (tetramethylammonium, hexamethonium, decaethonium, and susamethonium) have the same effects as SAD-128 (2-4). All of the studies indicated that some compounds, which have no oxime moiety and therefore have no reactivating potential, might bring beneficial effects on the reactivation of OP-inhibited AChE by oximes. According to these findings, perhaps we can find a new clue to the reactivation of soman-inhibited AChE through further study of enhancing the oxime-induced reactivation.

Up to now, we have noticed that nearly all effective compounds studied are inhibitors of the AChE, and there exist close relation between inhibitory potency and augmentative effect of those compounds. In the mean time, nearly all of the effective compounds studied are quaternary compounds with high toxicity, while the effects of tertiary compounds with relatively lower toxicity are seldom involved.

The purpose of this study is to study the effects of some tertiary compounds on the reactivatability of soman-inhibited human erythrocyte AChE, and compares them with a quaternary compound. The relationship between the anti-AChE properties of these compounds and their beneficial effects on the reactivation of the soman-inhibited AChE are discussed.

MATERIALS AND METHODS

Chemicals: Acetylthiocholine (ATCh) iodide, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB),

physostigmine salicylate (Phy), tetrahydroaminoacridine (THA, tacrine), decamethonium bromide (Deca) were purchased from Sigma. Huperzine A (Hup A), Carbaindoline (Carb), Toxogonin, and HI-6 (1-(4-carbamoylpyridinio)methoxymethyl-2-(hydroxyiminomethyl) pyridinium dicholoride monohydrate) were synthesized and provided by the Institute of Pharmacology and Toxicology (IPT, Beijing, China), and recrystallized before use. Soman was also obtained from our institute, and its purity was >98%. The other chemicals used were of analytical reagent grade.

Preparation of human erythrocytes: Suspensions of washed erythrocytes were prepared from blood units not older than 1 weeks obtained from a local blood bank. Blood samples of 1.0 ml were centrifuged for 5 min at 3500 rpm at 4_ and the supernatant was removed. The remaining erythrocytes were washed three times for 5 min at 3500 rpm in 1 ml 0.1 M phosphate buffer pH 7.4. After the last washing step, the cell were diluted to 1:3 suspension and stored at 4_.

Inhibition of AChE by four tertiary compounds and one quaternary compound: The concentration of the tested compounds giving 50% enzyme inhibition (IC_{50}) was determined by incubating with five or more different concentrations of each compounds (The final concentration of compounds ranged from 1_10^{-8} to 1_10^{-3} M) at 37_ and assaying for AChE activity according to Ellman et al(5) after 30 min. The enzyme activity was expressed as % of the activity observed in the absence of the tested compounds. The IC_{50} values were calculated by liner least-square regression of log enzyme activity vs concentration of the tested compound.

Effect of the five compounds on reactivation of soman-inhibited AChE by oximes: The experiments were performed in four steps (inhibition – aging – reactivation – assay) as Hallek and Szinicz reported in 1988 (4). For inhibition, 10 _l of 1:30 (v/v) human erythrocytes suspension were incubated with 10 _l of 2_$10^{-7.5}$ M soman in 0.1 M phosphate buffer pH 10 at 0_ for 30min, where aging is prevented, but not the phosphonylation of the enzyme (6). Then the samples were added to 10 _l of 0.1 M phosphate buffer pH 6.3 (final pH7.4) and were placed in a water bath at 37_ starting aging. At the same time, the respective compounds (effectors) were added (final concentration was 1 _$10^{-3, -4, -5, -6}$ M). After 0, 1, 3, 5, or 10min respectively, reactivation was started by adding HI-6 or toxogonin (final concentration was 1_10^{-4} M for HI-6 or 1_10^{-3} M for toxogonin). 20 min later, AChE activity was assayed. Before the assay, erythrocytes were washed three times with 0.1M pH 7.4 phosphate buffer to remove the excess oxime and the effector. Control runs were performed by replacing the effector (soman+oxime) or both the effector and oxime (soman) by 0.1 M phosphate buffer pH 7.4. All results are expressed as mean values ± SD at least four experiments.

Statistics: Statistical significance was determined by the use of one-way anova and differences were considered significant when $P<0.05$. Statistical evaluation was performed with SAS (release 6.03, SAS Institute).

RESULTS

Inhibition of human erythrocyte AChE by the five compounds

All five compounds caused an inhibition of human erythrocyte AChE with different inhibition potencies as judged by their IC_{50} values (Table 1). Among them, Phy, Carb and Hup A

showed much higher inhibiting effects on AChE, their IC_{50} values were two orders of magnitude higher than that of Deca. The results indicate a higher affinity of these tertiary compounds for AChE than the quaternary one studied. After washing the used compounds out of the reaction mixture, almost all AChE activity could recovery to more than 90% of normal values (data not shown).

Reactivation of soman-inhibited human erythrocyte AChE by the five compounds alone

After soman inhibited AChE without addition of any one of the five compounds, the enzyme activity were 8.5±3.1% of the normal value(mean±SD, n=4). The addition of some of the compounds at final concentrations from 1_10^{-3} M to 1_10^{-6} M caused slightly increases in AChE activity, but none of them were statistically significant of the reactivation of soman-inhibited enzyme (Table 2). The result indicated that the four tertiary reversible ChE inhibitors as well as the quaternary one alone did not have the ability to reactive the soman-inhibited AChE in vitro.

Effect of the effectors on the reactivation of soman-inhibited AChE by oximes at the start of aging

Table 3 shows the effects of the five effectors on the reactivatability by toxogonin of soman-inhibited AChE at the start of aging. When toxogonin was added alone at the start of aging, it caused little increase in AChE activity. But addition of each of the five compounds at the start of aging caused a marked increase in reactivation by toxogonin of soman-inhibited AChE. The five compounds were effective at different concentrations from 1_10^{-3} to 1_10^{-6} M, they exhibited statistically significant beneficial effects at 1_10^{-3} to 1_10^{-6} M. Decamethonium exhibited a statistically significant ($P<0.01$) beneficial effect only at a high concentration (1_10^{-3} M), and the maximum activity of reactivated enzyme was increase to 35.8±4.1% of the original AChE activity. The four tertiary compounds increased the AChE reactivating ability by toxogonin at lower concentrations than Deca does, among them Huperzine A was the most effective one. When addition of Hup A at a concentration of 1_10^{-5} M, the reactivation of soman-inhibited AChE by toxogonin could reached maximal level of 45.6±8.6% of the original AChE activity. Similar results were obtained when using HI-6 as reactivator (data not shown).

Effect of the effectors on the reactivation of soman-inhibited AChE by oximes after 1-10 min of aging

Figures 1 (a-b) show the effects of the effectors at concentrations that can bring the maximum effectiveness effect on the reactivation of soman-inhibited AChE by toxogonin or HI-6 with respect to the delay of oximes addition. When the four compounds (no Carbaindoline) were still added at the start of aging, but the addition of toxogonin or HI-6 delayed, the increases in the reactivatability of soman inhibited AChE obviously lowered. In the case of Hup A, when the addition of toxogonin or HI-6 delayed from the start of aging to after 3 min of aging, AChE activity rate decreased from 45.6±8.6% (by toxogonin) or 29.0±4.3% (by HI-6) to 19.7±2.6% or 18.2±4.2%, respectively. When toxogonin or HI-6 were added after 5 min to 10 min of aging, all of the compounds studied lost their beneficial effects on the reactivatability of the inhibited AChE.

DISCUSSION

The presented results demonstrate that the four tertiary compounds as well as a quaternary one are reversible AChE inhibitors of AChE, and all of them can enhance the reactivatability by toxogonin or HI-6 of soman-inhibited human erythrocyte AChE in vitro.

The comparison of the ability of such five compounds to improve the reactivatability of soman-inhibited AChE indicated that their inhibitory potency (IC_{50}) on AChE appeared to be a reliable indicator of the effective concentration. The four tertiary compounds with low IC_{50} value exhibited their beneficial effects at much lower concentrations than the quaternary one with relatively high IC_{50} values. Most of the effective compounds had clear maximum of effectiveness at the concentrations that were about 100 times higher than their respective IC_{50} value.

The majority of literature dealing with improving of reactivation by oximes of soman-inhibited AChE involves in studying the effects of numbers of quaternary compounds, such as decamethonium. Our work do confirms the effectiveness of decamethonium in increasing the reactivating ability by oximes of soman-inhibited AChE, in agreement with the results of previous investigations (1,3,4,7). However, like many other quaternary compounds, decamethonium has too high toxicity to be used in routine antidotal administration. On the other hand, as we know, quaternary compounds can not readily penetrate through blood-brain barrier (BBB) and get to the target part of brain to exert their effects. In contrast, tertiary compounds can easily penetrate BBB and reach the CNS to exert their antidotal effects in vivo, so the study of tertiary compounds effecting the reactivation of inhibited AChE might have much more practical significance.

In this study we have found that several tertiary compounds, such as Phy, Carb, THA and Hup A, all of them are reversible inhibitors of AChE, are even more effective in increasing the reactivatability than decamethonium. As reported by the publications, all four tertiary compounds we investigated have relatively low toxicity and have already been applied in clinical administration (8-9). So it is reasonable to assume that the tertiary compounds with relatively low toxicity might provide practical use by combination with quaternary oxime for improving the treatment of soman poisoning. In fact, in our further studies, we have noticed in soman-intoxicated mice that some of the tertiary AChE inhibitors do improve the therapeutic effect of toxogonin. This preliminary result is in agreement with our assumption.

Concerning the mechanism of action for the effectors, there are mainly two explanations in the literature(1-4): one is that the effectors might prevent the enzyme from phosphonylation, another one is that the effector might retard the dealkylation rate of the phosphonylation enzyme. From our results presented, we cannot obtain a certain explanation yet, but according to relationship between the anti-AChE properties of these compounds and their beneficial effects on the reactivation of the soman-inhibited AChE; we favor the first proposal. However, in our series similar experiments, we have found that all the compounds which can bring beneficial effects on the reactivatability of soman-inhibited AChE are reversible inhibitors of AChE, but not all the compounds which are reversible inhibitors of AChE can bring beneficial effects on the reactivatability of AChE and not all the compounds with same inhibition potencies have the same ability to improve the reactivation of enzyme. So the mechanism of the effects of all

compounds investigated may be not only explained as the reduction of the enzyme phosphonylation, and therefore we should consider for further studies about this.

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KEY WORDS

Soman, Acetylcholinesterase, Reactivation, Huperzine A, Decamethonium

FIGURES AND TABLES

(Figure head)

Figure 1(a-b). Effects of four reversible ChE inhibitors (Deca, Phy, Tacrine and Hup A) on the reactivation of soman-inhibited AChE by Toxogonin (a) or HI-6(b). AChE was incubated for 30 min in a solution containing soman at conditions preventing aging (0_, pH10). Before starting aging by changing the temperate (37_) and pH(7.4), the respective inhibitors were added. After 0, 1, 3, 5,10 min respectively, AChE was reactivated by toxogonin for 20 min. Control runs were performed by replacing the effector (soman + oxime) or both the effector and HI-6 (soman) by phosphate buffer (0.1 M, pH 7.4). All results are expressed as mean values ± SD of at least four experiments. Difference from control experiments (soman + oxime) was analysed by one-way anova (* P<0.05, ** P<0.01).

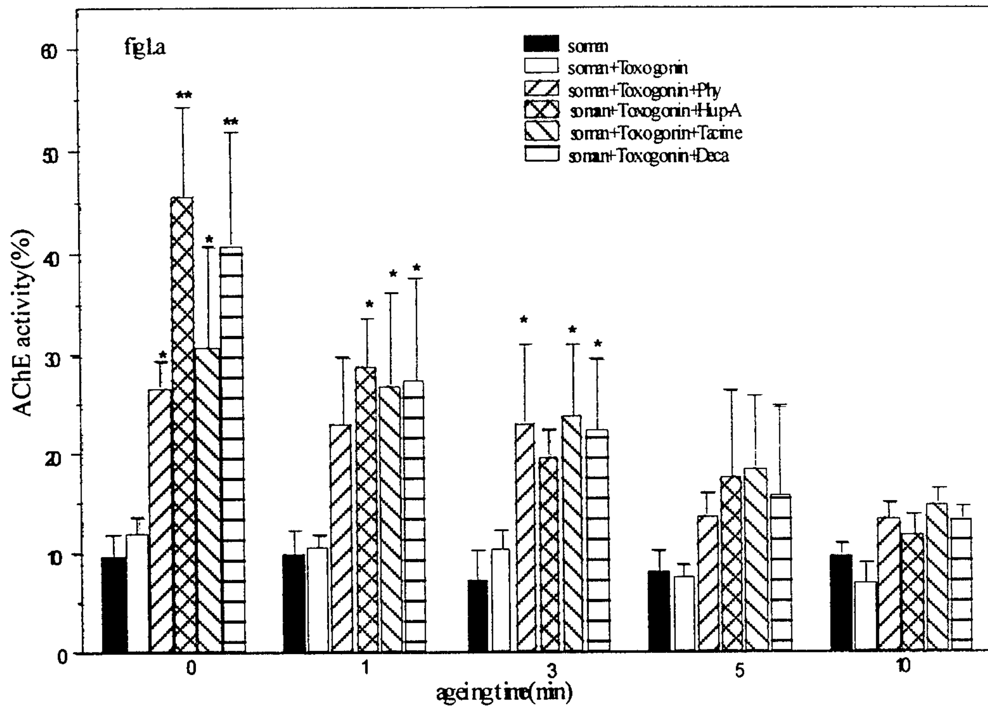
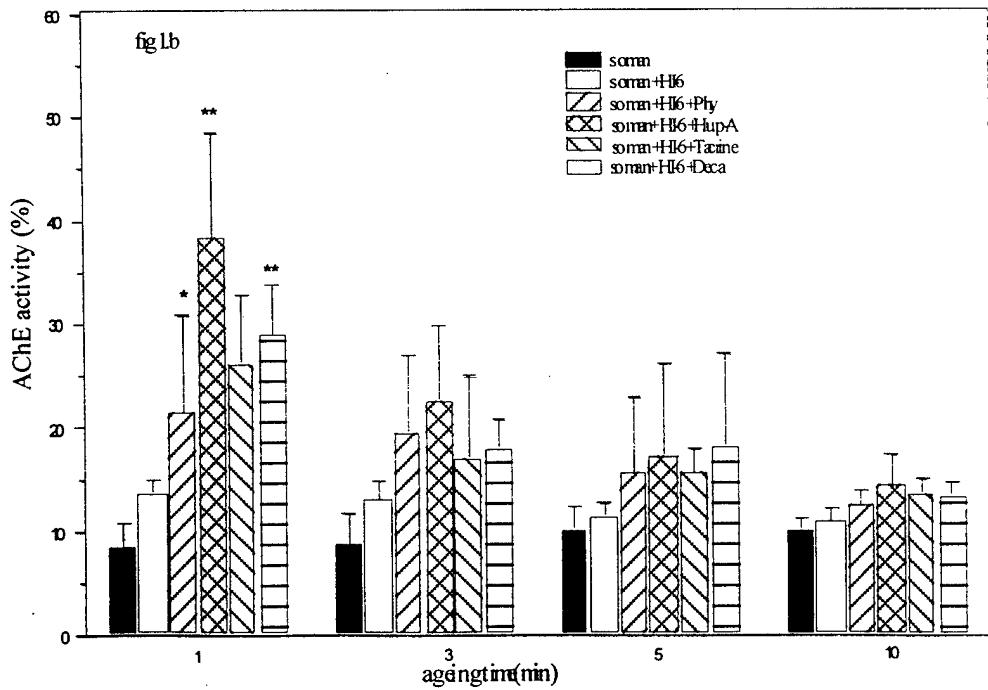


fig1.a
soman
soman+Toxogonin
soman+Toxogonin+Phy
soman+Toxogonin+HupA
soman+Toxogonin+Tacine
soman+Toxogonin+Deca
AChE activity(%)
ageing time(min)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soman
soman+HI6
soman+HI6+Phy
soman+HI6+HupA
soman+HI6+Tacine
soman+HI6+Deca
AChE activity (%)
ageing time(min)

13. CRITERIA FOR SELECTION OF HUMAN, ANIMAL AND PLANT PATHOGENS AND TOXINS TO BE INCLUDED IN A LIST OF THE BIOLOGICAL AGENTS AND TOXINS IN RELATION TO THE BTWC

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INTRODUCTION

During the sessions of the Ad-Hoc Group (AHG) of the States Parties to the Convention on the prohibition of the development, production and stockpiling of bacteriological (biological) and toxin weapons and on their destruction (BTWC), criteria and lists of human, animal and plant pathogens and toxins were discussed. The lists of agents and toxins in the Rolling text of the Protocol of the BTWC that were discussed in the section Definitions of Terms and Objective Criteria are for the use with specific measures, particularly for Article III, Compliance Measures, section A. Lists and Criteria (Agents and Toxins), section D. Declaration, paragraph 15 (Work with listed agents and toxins), section F, subsection I (Initial declaration and annual declarations).

This paper presents the results of five years of negotiations about one of the most important parts, on which all future work of the investigation of biological activities and all provisions of future Protocol of the BTWC will be based. These are the lists of biological agents and toxins that are selected according to current criteria for their inclusion in the lists. At the beginning of the negotiations, there were only 83 biological agents and toxins in the lists. There were 20 viruses, 9 bacteria, 3 rickettsia, two protozoa and one fungus in the list of human and zoonotic pathogens, 21 toxins, 19 animal and 18 plant pathogens. Many delegations submitted their Working papers during previous sessions for the evaluation and selection of some agents and toxins according to current criteria. The results of negotiations were very poor because that the bulk of pathogens and toxins were in square brackets, indicating that they needed further negotiations and considerations.

MATERIALS AND METHODS

The delegation of the Republic of Croatia submitted WP No. 356/Rev.1 at the fifteenth session of the AHG, which took place from 28 June to 23 July 1999. The intention was to show that all three current lists (human pathogens, toxins, and animal and plant pathogens) were valid at the moment by the existing criteria.. This proposal for the evaluation of pathogens and toxins induced and accelerated the negotiations in the section Definitions of Terms and Objective Criteria. Once we were able to show how the criteria applied to the existing lists, we suggested additional criteria, which could be used to add pathogens and toxins and further discriminate between them.

Since the human, animal and plant pathogens and toxin lists will be hard to define, we proposed several tables of pathogens and toxins along with important criteria for inclusion or exclusion. (See Tables 1-5.) These tables can serve as the basis for discussion and as help in defining the final list of bacteriological (biological) agents and toxins.

We used eleven criteria for the evaluation of human and zoonotic pathogens. We would like to point out that it is very hard to find in available literature all the data for the most important criterion, criterion number 1 (agents or toxins known to have been developed, produced, stockpiled or used as weapons). Therefore, we cannot be 100% sure that data for this criterion are correct. These lists can be of a great help in comparing pathogens and toxins and in making easier decision on including or excluding some particular pathogen and toxin in the lists. In the tables, the plus sign (+) signifies that pathogen or toxin satisfies the particular criterion for inclusion in the list. At the

same time the minus sign (-) signifies that pathogen or toxin does not satisfy criterion for inclusion in the list. The final column is the total of positive and negative answers.

The criteria are shown in Tables A through D, below. A high level of dissemination is a criterion we used in the evaluation of human pathogens and toxins. The key for producing large-scale respiratory infections is to generate an aerosol of suspended microscopic droplets, each containing one to thousands of bacterial or virus particles. A high level of dissemination or large-scale contamination or coverage of a large area with aerosol for respiratory exposure plays the main role in evaluation of particular agent or toxin.

The absence of effective prophylaxis or therapy, is an interesting criterion.. The existence of immunization and appropriate treatment against a particular agent is inversely proportional to the likelihood that the agent will be used. There are no effective prophylaxes or therapies for the majority of listed agents and toxins, if they are used as biological and toxin warfare agents. A full vaccination series for most diseases takes at least three months and in same cases up to one year. Hence it is difficult to imagine how a mass-vaccination would be effective against more than one disease.

Our opinion is that if some pathogen or toxin satisfies the greater part of the criteria, it should be recommended for inclusion in the list.. On the tables of human pathogens and toxins, you can see some biological agents and toxins, which are not in the current BTWC list. These additional agents and toxins are not recommended as new agents for including in the list. However, more detailed risk assessments and comparisons with pathogens and toxins on the existing list may give us more information so these agents can either be added or dropped from consideration.

The evaluation of agents and toxins according to existing criteria is valid only under assumption that all criteria have equal status. We have to agree and make the final decisions as to whether all the criteria are valid and have the same value in evaluating particular pathogen or toxin. We are not sure that all criteria are equal for the final evaluation. Perhaps we should consider it and request an opinion from the scientists and experts who will give the best opinion on validity of certain criteria and maybe even sort them by value or points. The criteria we use for evaluating the biological agents and toxins are based on the characteristics outbreaks of infectious diseases in "natural" forms. Genetically engineered and modified bacteria and viruses present a difficult problem. New criteria reflecting new characteristics of bacterial and viral strains enhanced for infectivity, transmissibility, virulence and antibiotic resistance should be inserted.

The additional list of criteria for toxins and a list of toxins were prepared to help in final discussions. These additional criteria for toxins are actually a combination of existing and additional criteria and are not recommended as new criteria. It can be used for more detailed risk assessments and for comparisons of the toxins on the list. In this case, the lower the total number, the more dangerous is the toxin as a toxin warfare agent. In the additional list of criteria for toxins, we can see at a glance which criterion is unimportant: Ease of decontamination. For toxins, decontamination would be relatively unimportant, but fungal toxins are extremely difficult to decontaminate and once an area is contaminated (infected), it can take years of hard cleaning to eliminate the toxins, especially if delivered with fungal spores.

Thetoxicity of toxins should be evaluated with caution; agents that are nonlethal but militarily incapacitating should be included. Lethality alone is not an appropriate criterion on which to base a toxin's potential as an agent. For evaluation of toxins, we prefer to include those toxins that act primarily as incapacitating agents, because these toxins have high potencies and they represent a significant trend for the future. For example, Staphylococcal entertoxin B (SEB), a so-called super-antigen, is one of the most potent agents for incapacitating because it can cause illness at extremely

low doses,but relatively high doses are required to kill. Trichothecene-Mycotoxins, Batrachotoxins and Brevetoxins can be included in this list. If on the other hand we included only those lethal toxins, we would be underestimating the number and potential of toxins as agents.

Table A. CRITERIA FOR SELECTION OF HUMAN AND ZOONOTIC PATHOGENS AND TOXINS

1. Agents or toxins known to have been developed, produced, stockpiled or used as weapons
2. Likely methods and high level of dissemination or cover a large area as aerosol, spores in aerosol, sabotage (food and water supply) and infected vector
3. Low infective/toxic dose
4. High level of morbidity and short incubation or latent period
5. High level of transmissibility and/or contagiousness
6. Infection or intoxication by variety of route, especially by respiratory route
7. High morbidity, incapacity or mortality rates
8. Stability in the environment
9. No effective or cost-effective prophylactics, protection or treatment available
10. Short incubation period and/or difficult to diagnose/identify at an early stage
11. Ease of production and dissemination

Table B. ADDITIONAL CRITERIA FOR TOXINS

Toxicity

1= Lethal dose (LD_{50}) in the 10^{-9} g/kg range

10= Lethal dose (LD_{50}) in the 10^{-3} g/kg range

Onset

1= Minutes to hours onset

10 = Hours or days to onset

Level of incapacity or mortality

1= Predominately incapacitating

10= Predominately lethal

Likely methods of dissemination

1= Toxin could be aerosolised and delivered to cover large areas (large-scale dissemination). Toxin could be used in sabotage for contamination food and water.

10= Toxin could not be aerosolised and delivered to cover large areas. Toxin could be difficult to use in sabotage.

Stability in environment/storage

1= Extremely stable in storage and environment

10= Unstable in environment or requires special storage conditions

Ease of decontamination

1= Extremely difficult to decontaminate after a toxin aerosol attack

10= Decontamination would be relatively unimportant and general decontamination procedures effectively destroy toxin

Ease of production and transportation

1= Toxin can be easily produced in large quantities - low technology, low cost, widely available (fermentation)

10= Toxin that is very difficult to produce in weaponizable quantities - high cost, only available to specialised teams (solid phase synthesis of >100 amino acid polypeptides, advanced genetic manipulation).

Table C. CRITERIA FOR ANIMAL PATHOGENS

1. Agents known to have been developed, produced or used as weapons.
2. Agents which have severe socio-economic and/or significant adverse human health impacts to be evaluated against a combination of the following criteria:

a. High morbidity and/or mortality rate
b. Short incubation period and/or difficult to diagnose/identify at an early stage
c. High transmissibility and/or contagiousness
d. Lack of availability of cost effective protection/treatment
e. Low infective/toxic dose
f. Stability in the environment
g. Ease of production

Table D. CRITERIA FOR PLANT PATHOGENS

1. Agents known to have been developed, produced or used as weapons.
2. Agents which have severe socio-economic and/or significant adverse human health impacts, due to their effect on staple crops, to be evaluated against a combination of the following criteria:

a. Ease of dissemination (wind, insects, water, etc.)
b. Short incubation period and/or difficult to diagnose/identify at an early stage
c. Ease of production
d. Stability in the environment
e. Lack of availability of cost effective protection/treatment
f. Low infective dose
g. High infectivity
h. Short life cycle.

RESULTS AND DISCUSSIONS

The results of negotiations on the twentieth session of AD-Hoc Group are presented in the tables that follow.

Table E. Human and Zoonotic Pathogens

Viruses	Bacteria
1. Crimean-Congo haemorrhagic fever virus	1. Bacillus anthracis
2. Eastern equine encephalitis virus	2. (Brucella abortus(
3. Ebola virus	3. Brucella melitensis
4. Sin Nombre virus	4. (Brucella suis(
5. Junin virus	5. Burkholderia mallei
6. Lassa fever virus	6. Burkholderia pseudomallei
7. Machupo virus	7. Francisella tularensis
8. Marburg virus	8. Yersinia pestis
9. Rift Valley fever virus	9. Coxiella burnetii
10. Tick-borne encephalitis virus	10. Rickettsia prowazekii

11. Variola major virus (Smallpox virus)
12. Venezuelan equine encephalitis virus
13. Western equine encephalitis virus
14. Yellow fever virus
15. Monkeypox virus

11. Rickettsia rickettsii

(Protozoa
1. Naegleria fowleri
2. Naegleria australiensis(

Table F. Animal Pathogens

Bovine pathogens
1. (Contagios bovine (pleuropneumonia)/ (Mycoplasma mycoidey var. mycoides(
2. (Foot and mouth disease virus(
3. Rinderpest virus
4. (Vesicular stomatitis virus(

Ovine pathogens
5. (Peste des petitis ruminants virus(
6. (Blue tongue virus(

Swine pathogens
7. African swine fever virus
8. (Classical swine fever virus (Hog cholera virus)(
9. (Teschen disease virus (Porcine enterovirus type 1)(

Avian pathogens
10. (Avian influenza virus (Fowl plague virus)(f.sp. tabacina (Adam) skalicky(
11. (Newcastle disease virus(

Equine pathogens
12. (African horse sickness virus(

Table G. Plant Pathogens

Cereal pathogens
1. (Puccinia graminis
2. Tilletia indica
3. (Claviceps purpurea(

Sugar cane pathogens
4. (Sugar cane Fiji disease virus(
5. Xanthomonas albilineans

Cash crop pathogens
6. Colletotrichum coffeanum var. virulans
7. (Erwinia amylovora(
8. (Ralstonia solenacearum(
9. (Xanthomonas campestris pv citri(
10. (Sclerotinia sclerotiorum(
11. (Peronospora hyoscyami de Bary

Forest pathogens
12. (Dothistroma pini (Scirrhia pini)(

Table H.. Toxins

Bacteriotoxins
1. Botulinum toxins
2. Clostridium perfringens toxins
3. Staphylococcal enterotoxins
4. Shigatoxins

Phycotoxins
5. Anatoxins
6. Ciguatoxins
7. Saxitoxins

Mycotoxins
8. Trichothecene toxins

Phytotoxins
9. Abrins
10. Ricins

Zootoxins
11. Bungarotoxins

The list of viruses, as a part of list of human pathogens, consists of the15 viruses listed above. The list of human pathogens includes bacterial agents. I think that Brucella suis and Brucella abortus will be deleted during further negotiations. Also, two protozoa, Naegleria fowleri and Naegleria australiensis, will be deleted. The final list of toxins consists of 11 toxins.

In the list of animal pathogens, two agents Rinderpest virus and African swine fever virus, have been already resolved. After a long time Blue tongue disease virus was returned in the list. All the others are in square brackets and need further negotiations and considerations. My opinion is that: Classical swine fever virus, Newcastle disease virus, Avian influenza virus, Foot and mouth virus and Vesicular stomatitis virus should be kept in the list. The Croatian delegation proposed inclusion of Nipah virus in the list of animal pathogens and this was kept in the list about one year. However, it was deleted on the nineteenth session. The reason for the deletion of this agent was because the outbreak of Nipah encephalitis covered only a small part of the world and, for the time being, does not satisfy the criteria for inclusion in the list.

In the list of plant pathogens you can see Tilletia indica, Xathomonas albilineans and Colletotrichum coffeanum var. virulans that are resolved, but all other pathogens need further negotiations and considerations. In my view, Puccinia graminis and Xathomonas compestris citri should be kept in the list.

CONCLUSIONS

From this presentation you can see it is very hard to make a final decision on criteria, and, hence, on the final list of agents and toxins for the future needs of Protocol to the BTWC based on these criteria. We propose that lists and criteria for agents and toxins should be well studied and that opinions by scientists and experts should be obtained.

The list is not exhaustive; it does not exclude those unlisted microbial or other biological agents or toxins potentially can be used as weapons or vectors (such as pests, arthropods and helminthes). The microorganisms enumerated in the lists of human, animal and plant pathogens do not include

live-attenuated strains, which have been registered as such in official culture collections or are internationally recognized as such.

Further consideration will be needed to give to microorganisms carrying nucleic acid sequences coding for pathogenic properties of listed agents and toxins and nucleic acid sequences coding for toxins.

Any State Parties of the BTWC may propose modifications to the lists. The Executive Council of the OPBTW shall review such proposed modifications to the list of agents and toxins. Any changes to the list shall be made in accordance with Articles III. and XIV. of the Protocol. In reviewing the lists of agents and toxins the Executive Council shall consider current criteria as well as:

- Scientific and technological developments that may affect the potential of individual agents and toxins for use as weapons.
- Effects of potential inclusion or exclusion of an agent or toxin in the list on scientific and technical research and development.

And finally, the main goal of this presentation is to induce discussions among and from scientists and experts who are involved with biological and toxin warfare agents or weapons as part of their job. It seems that our group of scientist and experts in the CBMTS members are not sufficiently included in negotiations in Ad-Hoc Group of the BTWC within their delegations.

KEY WORDS
human, animal and plant pathogens, and toxins, BTWC.

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FIGURES AND TABLES

These are in a separate file.

Table 1. Human and zoonotic pathogens (viruses) assessment according to criteria for selecting pathogens as BW

Viruses	Weapo-nized	High level of dissemi-nation	Low infec-tion dose	High level of morbi-dity	High con-tagiousness (transmiss-ibility man to man)	Infection by variety of route (respira-tory route)	High level of incapacity / mortality	Stability in the environ-ment	Difficulty of detection / identifi-cation	No effective prophylaxis and/or therapy	Ease of produ-ction	Totals +/-
:rimean-Congo HF virus	+	+	+	+	-	+	+	+	+	+	-	9/2
:EE virus	+	+	+	+	-	-	+	-	+	+	-	7/4
:bola virus	+	+	+	+	+	+	+	-	+	+	+	10/1
in Nombre virus	-	+	+	+	-	+	+	-	+	+	-	7/4
[antaan virus	+	+	+	+	-	+	-	+	+	+	-	8/3
unin virus	-	+	+	+	-	+	+	-	+	+	-	7/4
,assa fever virus	+	+	+	+	-	+	+	+	+	+	-	9/2
1achupo virus	-	+	+	+	+	+	+	-	+	+	-	8/3
1arburg virus	+	+	+	+	+	-	+	-	+	+	-	8/3
:ift Valley fever virus	+	+	+	+	-	-	-	+	+	-	-	6/5
'ick-borne enceph. virus	+	+	+	+	-	-	+	+	+	-	-	7/4
'ariola major virus	+	+	+	+	+	+	+	+	+	-	+	10/1
'EE virus	+	+	+	+	-	+	+	-	+	-	-	7/4
VEE virus	+	+	+	+	-	+	+	-	+	-	-	7/4
'ellow fever virus	+	+	+	+	-	+	+	-	+	-	+	8/3
1onkeypox virus	-	+	+	+	+	+	+	+	+	-	-	8/3
:hikun-Gunya fever v. (CHIK)	-	+	+	-	-	+	-	-	+	+	-	5/6
)engue fever virus	+	+	+	+	-	-	-	-	+	-	-	5/6
)msk HF virus	-	+	+	+	-	-	-	-	+	-	-	4/7

Table 2. Human and zoonotic pathogens (bacteria, rickettsiae, protozoa and fungi) assessment according to criteria for selecting pathogens as BW

cteria Rickettsiae Protozoa Fungi	Weaponized	High level of dissemination	Low infection dose	High level of morbidity	High contagiousness (transmissibility man to man)	Infection by a variety of route (respiratory route)	High level of incapacity or mortality	Stability in the environment	Difficulty of detection/ identification	No effective prophylaxis (vaccination)	No effective therapy (anti-microbial)	Ease of production	Totals +/-
						Bacteria							
lus anthracis	+	+	+	+	-	+	+	+	+	-	-	+	9/3
lla abortus	+	+	+	+	-	-	-	+	+	+	-	+	8/4
lla melitensis	+	+	+	+	-	-	+	+	+	+	-	+	9/3
lla suis	+	+	+	+	-	-	-	+	+	+	-	+	8/4
holderia domonas) mallei	+	+	+	+	-	+	+	+	+	+	+	+	11/1
holderia (Pseudom.) lomallei	+	+	+	+	-	+	+	+	+	+	+	+	11/1
nydia psittaci	+	+	+	+	-	+	-	+	+	+	-	-	8/4
isella tularensis	+	+	+	+	-	+	+	-	+	-	-	+	8/4
nia pestis	+	+	+	+	+	+	+	-	+	-	-	+	9/3
						Rickettsiae							
lla burnetti	+	+	+	+	-	+	-	+	+	-	-	-	7/5
ttsia prowazekii	+	+	+	+	-	-	+	-	+	+	-	-	7/5
ttsia rickettsii	+	+	+	+	-	+	+	-	+	+	-	-	8/4
						Protozoa							
leria fowleri	-	+	-	+	-	+	+	-	-	+	-	+	6/6
leria australiensis	-	+	-	+	-	+	+	-	-	+	-	+	6/6
						Fungi							
idioides immitis	-	+	-	-	-	+	+	+	+	+	-	+	7/5
plasma capsulatum	-	+	-	+	-	+	-	+	+	+	+	+	8/4
rdia asteroides	-	+	-	-	-	+	+	+	+	+	-	+	7/5

Table 3. Toxin assessment according to criteria for selecting toxins as TW

Toxin/Bioregulator	Weapon ized	High toxic ity	High morbi dity	Intoxication by variety of route-respiratory route	High level of incapacity/ mortality	No effective prophylaxis/ therapy	Stability in the environme nt	Difficulty of detection/ identification	Ease of product ion	Total s +/-
Abrin	-	+	+	+	+	+	+	+	+	8/1
Aflatoxins	+	-	+	+	+	+	+	+	+	8/1
Anatoxin A	+	+	+	+	+	+	-	+	+	8/1
Batrachotoxin	-	+	+	+	+	+	-	+	-	6/3
Botulinum toxins	+	+	+	+	+	-	+	+	+	8/1
Brevetoxins	-	+	+	+	+	+	+	+	-	7/2
Bungarotoxins	+	+	+	+	+	-	-	+	-	6/3
Centruroides toxins	-	+	+	+	+	-	+	+	-	6/3
Ciguatoxin	-	+	+	+	+	+	-	+	-	6/3
Cyanginosins/Microcystins	-	+	+	+	+	+	-	+	-	6/3
Diphtheria toxin	-	+	+	+	+	-	-	+	-	5/4
Modeccin	-	+	+	+	+	+	+	+	+	8/1
Palytoxin	-	+	+	+	+	+	+	+	-	7/2
Ricin	+	+	+	+	+	+	+	+	+	9/0
Saxitoxin	+	+	+	+	+	+	+	+	-	8/1
Staphylococcal enterotoxins (SEB)	+	+	+	+	+	+	+	+	+	9/0
Shigatoxin	+	+	+	+	+	+	+	+	+	9/0
Tetanus toxin	+	+	+	+	+	-	+	+	+	8/1
Tetrodotoxin	+	+	+	+	+	+	+	+	-	8/1
Toxins of Cl. perfrigens	+	+	+	+	+	+	-	+	+	8/1
Trichotecene Mycotoxins (T2,DON,HT2)	+	+	+	+	+	+	+	+	+	9/0
Verrucologen	-	+	+	+	+	+	-	+	-	6/3
Viscumin	-	+	+	+	+	+	+	+	+	8/1
Volkensin	-	+	+	+	+	+	-	+	-	6/3
Endothelin/Sarafotoxin	-	+	+	+	+	+	+	+	+	8/1

Table 4. Toxin assessment according to additional criteria (the lower the total number, the more dangerous the toxin as TW)

Toxin/ Bioregulator	Toxicity	Onset	Level of incapacity/ mortality	Likely methods of dissemination	Stability in the environment/ storage	Ease of decontamina tion	Ease of production	Totals
.brin	2	6	5	5	5	5	1	29
.flatoxins	7	8	5	5	5	1	3	34
.natoxin A	5	1	6	7	6	8	3	36
atrachotoxin	3	2	3	4	9	8	8	37
otulinum toxins	1	3	7	3	2	6	1	23
revetoxins	6	6	2	4	2	3	8	31
ungarotoxins	3	4	6	5	8	7	8	41
entruroides toxins	3	4	6	5	2	5	8	33
iguatoxin	3	7	6	6	8	5	9	44
yanginosins/Microcystins	5	2	5	3	7	7	8	37
iphtheria toxin	2	3	6	5	5	7	6	34
Iodeccin	3	6	5	4	5	5	1	29
alytoxin	2	4	8	3	5	3	9	34
icin	3	6	8	3	2	5	1	28
axitoxin	3	2	8	3	3	7	5	31
taphylococcal enterotoxins (SEB)	4	6	2	2	3	5	2	24
higatoxin	1	4	2	3	3	7	2	22
etanus toxin	1	9	8	4	3	7	2	34
etrodotoxin	3	4	5	3	5	5	9	34
oxins of Cl. perfrigens	3	6	8	3	3	7	3	33
richotecene Mycotoxins (T2,DON,HT2)	7	2	7	2	1	2	2	23
errucologen	3	7	6	5	6	6	3	36
iscumin	3	6	5	5	6	6	1	32
olkensin	4	5	7	6	7	5	4	38
ndothelin/Sarafotoxin	6	1	7	2	3	5	4	28

Table 5. Animal pathogens assessment according to criteria for selecting pathogens as BW

Animal pathogens	Weapo-nized	Severe socio-economic/ human health impacts	High morbidity/ mortality rates	Short incuba tion period	High transmissi bility/ contagiou sness	Low infective/t oxic dose	Difficult to diagnose/ identify at an early stage	Stability in the environ-ment	Lack of availabilit y of cost-effective protection /treatment	Ease of producti on	Total s +/-
Viruses											
frican swine fever virus	-	-	+	+	+	+	+	+	+	-	7/3
vian influenza virus	+	-	+	+	+	+	+	+	+	-	8/2
amel pox virus	-	-	+	+	-	+	+	+	+	-	6/4
lue tongue virus	-	-	-	+	-	+	+	+	-	-	4/6
lassical swine fever virus	+	+	+	+	+	+	+	+	-	-	8/2
oot and mouth virus	-	+	+	+	+	+	-	+	-	+	7/3
ewcastle disease virus	+	+	+	+	+	+	+	+	-	-	8/2
est des petits minants virus	-	+	+	+	+	+	+	+	-	-	7/3
inderpest virus	+	+	+	+	+	+	+	+	-	-	8/2
orcine enterovirus type 1	-	-	+	+	+	+	+	+	+	-	7/3
esicular stomatitis virus	-	-	+	+	+	+	+	+	+	-	7/3
frican horse sickness virus	-	-	+	+	-	+	+	-	+	-	5/5
umpy skin disease virus	-	-	-	+	-	+	+	+	-	-	4/6
ipah swine encephalitis virus	-	+	+	+	+	+	+	+	+	-	8/2
Mycoplasmas											
ontagious bovine (pleuropneum.) (M. ycoides var. mycoides type SC) (CBPP)	-	-	-	-	+	+	+	-	-	+	4/6
ontagious caprine (pleuropneum.) (M. priculum var. capri pneumoniae type F38) CPP)	-	-	-	-	+	+	+	+	-	+	5/5

Table 6. Plant pathogens assessment according to criteria for selecting pathogens as BW

Plant pathogens	Weapon ized	Severe socio-economic/ human health impacts	Short incuba tion period	Ease of dissemination (wind, insects, water, etc.)	Short life cycle	Low infective dose and infectivity	Difficulty diagnose/ identify at an early stage	Stability in the environ-ment	Lack of availability of cost-effective protection/ treatment	Ease of product ion	Tota ls +/-
				Fungi							
olletotrichum coffeanum var. virulans	-	-	+	+	-	+	+	+	+	+	7/3
othistroma pini (Scirrhia pini)	-	-	+	+	-	+	+	+	+	-	6/4
laviceps purpurea	-	+	+	+	-	-	+	-	+	-	5/5
eronospora hyoscyami de Bary	-	-	+	+	-	+	+	+	+	-	6/4
uccinia graminis	+	+	+	+	-	+	+	+	+	+	9/1
uccinia striiformis (P. glumarum)	-	+	+	+	-	+	+	+	-	+	7/3
yricularia oryzae	+	+	+	+	-	+	+	+	+	+	9/1
clerotinia sclerotiorum	-	+	+	+	-	+	+	-	+	-	6/4
illetia indica	+	+	+	+	-	+	+	+	+	+	9/1
stilago maydis	-	+	+	+	-	+	+	+	+	+	8/2
				Bacteria							
rwinia amylovora	-	+	+	+	-	+	+	-	+	+	7/3
alstonia solanacearum	-	-	+	+	-	-	+	+	-	-	4/6
santhomonas albilineans	-	+	+	+	-	+	+	-	-	+	6/4
anthomonas campestris pv. citri	-	+	+	+	-	+	+	-	-	+	6/4
anthomonas campestris pv. oryzae	-	+	+	+	-	+	+	-	-	+	6/4
				Viruses							
ugar cane Fiji disease virus	-	+	+	-	-	+	+	-	+	+	6/4

Table 6. Plant pathogens assessment according to criteria for selecting pathogens as BW

Plant pathogens	Weaponized	Severe socio-economic/ human health impacts	Short incubation period	Ease of dissemination (wind, insects, water, etc.)	Short life cycle	Low infective dose and infectivity	Difficulty diagnose/ identify at an early stage	Stability in the environment	Lack of availability of cost-effective protection/ treatment	Ease of production	Totals +/-
				Fungi							
olletotrichum coffeanum var. virulans	-	-	+	+	-	+	+	+	+	+	7/3
othistroma pini (Scirrhia pini)	-	-	+	+	-	+	+	+	+	-	6/4
laviceps purpurea	-	+	+	+	-	-	+	-	+	-	5/5
ɜronospora hyoscyami de Bary	-	-	+	+	-	+	+	+	+	-	6/4
uccinia graminis	+	+	+	+	-	+	+	+	+	+	9/1
uccinia striiformis (P. glumarum)	-	+	+	+	-	+	+	+	-	+	7/3
yricularia oryzae	+	+	+	+	-	+	+	+	+	+	9/1
clerotinia sclerotiorum	-	+	+	+	-	+	+	-	+	-	6/4
illetia indica	+	+	+	+	-	+	+	+	+	+	9/1
stilago maydis	-	+	+	+	-	+	+	+	+	+	8/2
				Bacteria							
rwinia amylovora	-	+	+	+	-	+	+	-	+	+	7/3
alstonia solanacearum	-	-	+	+	-	-	+	+	-	-	4/6
santhomonas albilineans	-	+	+	+	-	+	+	-	-	+	6/4
anthomonas campestris pv. citri	-	+	+	+	-	+	+	-	-	+	6/4
anthomonas campestris pv. oryzae	-	+	+	+	-	+	+	-	-	+	6/4
				Viruses							
ugar cane Fiji disease virus	-	+	+	-	-	+	+	-	+	+	6/4

14. THE SCIENTIFIC REVIEW OF CBMTS - INDUSTRY I

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It is great pleasure to be here in Spiez, a remarkably beautiful place and in the AC Laboratory where the CBMTS series was born and started, and to have the opportunity to address you at the opening session of CBMTS-III.

Distinguished Mister President of the Organizing Committee of CBMTS-III, dear colleagues and friends, allow me to present a short scientific overview of the symposium, CBMTS - Industry I or "Ecoterrorism: Chemical and Biological Warfare without Chemical and Biological Weapons", which took place in Zagreb and Dubrovnik in October 1998.

CBMTS-Industry I, organized as a regional symposium, was very successful and expanded beyond the regional boundaries. The topic raises a number of questions, which can be interpreted in various ways. The CBMTS-Industry I dramatically demonstrated that extremely dangerous conditions could exist within the complexes of all chemical, petrochemical, pharmaceutical and other related industries. These dangerous conditions, which include the use of legal but very toxic chemicals on a large scale, when triggered or aggravated by acts of terrorism, sabotage, combat or a large-scale incident or accident could lead to catastrophes. These could equal or exceed consequences expected of actual chemical or biological warfare attacks. There are no specific legal or technical safeguards that would prevent these acts from occurring, and therefore, communities must be very vigilant and thorough in their preparations to prevent and mitigate catastrophes.

The Symposium "CBMTS-Industry I" consisted of 72 scientific papers that were presented by oral and poster presentation. These papers were divided into 11 sessions. In addition to the scientific papers, several demonstrations were held on the first day of the Symposium that proved our thesis of chemical and biological warfare without chemical and biological weapons: the highly successful exercise "Kutina 98", which showed the possible effects of the attack on the mineral fertilizer plant "Petrokemija" Kutina, the presentation by CROSCO of its system to fight and cap oil well fires, and the presentation of the counter-terrorism equipment of the Canadian company Irvine Aerospace.

The session **"Understanding, Prevention and Minimization of Industrial Accident Consequences"**, chaired by Dr. David Moore, brought together experts from eight countries to present their experiences in industrial process engineering, transportation and storage of dangerous materials, safety, computer risk modeling, toxicology, evacuation procedures, medical treatment and care of large number of poisoned and wounded persons. They also described the plans, programs, processes and organizations that focus on assessment, planning and training for quick responses to potential sudden releases of large quantities of toxic chemical substances either as accidents or as strategic, objectives of war or tactical objectives of terrorists. The lectures of this session showed the vulnerability of chemical, oil, petrochemical, pharmaceutical, biological and other industries that use large quantities of dangerous and toxic chemical substances in their production processes.

Croatian authors presented experiences from the war and all the horrors and possible consequences of such industrial accidents caused by the war. In many countries, it is recognized that attacks on large chemical industries are strategic wartime objectives, however these same industries are also ideal tactical targets for terrorist. Lectures of US authors described procedures and methods of assessment of danger and consequences of terrorist attacks on large industrial facilities aroused a great interest. In the US, the Public Health Service uses a 10-step process in assessing the terrorist threat, target vulnerability and consequence management requirements as they relate to large industrial facilities. An understanding of the dynamics of timetables for responses permits better coordination between first responders and medical care providers and ultimately benefits the victims of the disaster.

Session "**Epidemiology, Biological Terrorism and Biological Warfare**" chaired by Dr. Sergey Netesov, and Dr. Leo Laughlin contained the most papers. The Croatian authors in that section showed their experiences in assessment of possible consequences of potential usage of biological warfare agents and procedures for quick response. The papers aroused a great interest presenting the role of epidemiological studies in possible usage of biological warfare agents, new diagnostic methods and mathematical methodology for predicting the infectivity of viruses in humans from animal studies. Biological warfare agents do not necessarily have to be exotic diseases. Epidemic/Endemic viral diseases could be used by terrorists and could go unnoticed unless public

health investigators use molecular biology techniques to identify and define the causative agents in an outbreak. Dr. Netesov suggested an organization of a series of labs that can be used as rapid diagnosis and detection centers on an international cooperative basis. The lecture that showed possibilities of modification of Ebola virus, as one of the most dangerous biological warfare agent, for terrorist usage aroused special interest. Namely, it is known that this virus cannot be converted to a highly contagious respiratory agent. However, Ebola virus may change its tropism to respiratory lining cells and its biological properties may be affected under natural, ecological and other active influences. With such changes, Ebola virus could be transformed to an extremely contagious and dangerous form and could become a most dangerous weapon for a terrorist use today.

The papers in the Session "**Emergency Management,**" chaired by Dr. Brian Davey were divided into three main topic areas. The first group described industrial accidents and incidents, facilities and experiences of Croatian authors. Discussions raised by these papers revealed the need for a clear analysis of the differences between standard safety and accident prevention and response in chemical industries and the additional considerations that arise when facilities are threatened by conflict or identified as a potential terrorist targets.

The discussions covered the possibilities that chemical and biological technologies offer terrorists and included a wide range of examples from the assassination of political targets using ricin toxin to the contamination of foodstuffs and drinks. The motives, potential victims and nature of attacks were described in a study that analyzed 200 incidents and accidents that happened during the last 20 years worldwide. Response to such incidents and the need to supplement training and organization of first line response groups was highlighted. Canadian authors presented a new method of decontamination of skin and skin injury. The new Canadian decontaminant RSDL was presented.

The session titled "**Chemical Warfare Agents,**" chaired by Dr. Rudolf Portmann, included papers that dealt with the health effects of chemical warfare agents, various methods of detection, exposure monitoring, and destruction and sequestering of chemical warfare agents. The methodology for acetylcholinesterase. (AChE) activity determination, with the aim to standardize one widely accepted method for usage in occupational health and clinical toxicology was also discussed. The comprehensive work on late effects of nerve agent done in the US showed that there were few persistent effects after non-symptomatic nerve agent exposure. Several well-accepted papers about antidotal therapy nerve agent poisoning, destruction of stored Lewisite and benefits of zeolites for decontamination and destruction of hazardous chemical materials in environment were also presented.

The poster session chaired by Prof. Ladislav Palinka presented 13 scientific papers relating biological and chemical weapons, infectious diseases, measures and actions for prevention of industrial accidents caused by the war, and a poster presenting a new antidote against intoxication of nerve chemical warfare agent soman.

The majority of participants showed a special interest in the lecture of Dr. Murray Hamilton from Canada. the session chair for "**Toxins**". This scientific paper summarized all the characteristics of toxin warfare agents that are considered a great threat nowadays. He also discussed new methods of syntheses and production of toxins in large quantities for military usage. This report categorized toxins as mid-spectrum agents, especially with respect to the similarities and differences to classical chemical and biological agents. This lecture presented all known toxicological characteristics of toxins, clinic descriptions of intoxication and unfortunately all the impossibilities in early stage detection, non-effective medical treatment and therapy.

The section under title "**Legal**", chaired by Dr. Barry Kellman and co-chaired by Elizabeth French, noted the possibilities of legal sanctioning of military attacks on industrial facilities that deal with large quantities of dangerous and toxic chemical substances. If the intent is to cause mass casualties with a large number of poisoned and dead people, and destroying wildlife and environment, should such an attack be considered as chemical warfare or not.

The revision and customization of international warfare regulations do not always keep pace with actual events and are often affected by political, ideological, economical and other interests and contradictions within the international community. The papers in the field of regulation of industrial accidents caused by military actions pointed out a possible expansion of the role of UN in international negotiations. Our thesis and trademark "Chemical and biological warfare without chemical and biological weapons" has now been recognized by the international diplomacy.

The OPCW representative recognized this legal problem and showed great interest for further discussions and maybe comprehension of our thesis under decisions of CWC. How difficult it is to change international warfare law regulations was demonstrated to us by the long duration of negotiations for Conventions such as CWC, and, how much time will be needed for finishing the very difficult negotiations in Ad-hoc Group of States Parties to the BTWC. However, this thesis is a reality today and consequently this interest is justified.

"CBMTS - Industry I," to our great pleasure, aroused a great interest in the international scientific field. The areas especially interested are those in industrial process engineering, transport and stockpiling of dangerous chemical substances, computer risk modeling, prevention of industrial accidents and incidents, toxicology of chemical, biological and toxin warfare agents, medical treatment and care of large numbers of poisoned and wounded persons.

"CBMTS-Industry I" also included very successful exercises demonstrating practical aspects of responses "Eco-Terrorism, Chemical and Biological Warfare without Chemical and Biological Weapons" or "Chemical and Biological Warfare with Conventional Weapons".

One of the main goals of this symposium was to show the real threat of industrial accidents caused by wars and terrorist actions, sabotage, accidents and incidents in the industry, and to consider scientific and professional measures and actions to reduce and eliminate such consequences for civilians and environment. The effects of such disasters could be achieved by attacking chemical and petrochemical plants, oil industries, pharmaceutical plants, biotech industries and other industries that deal with large quantities of very toxic substances. All of these may have potentials for Trans-boundary Effects of Industrial Accidents (Convention, UN, Helsinki, 1992).

Highly respected colleagues and friends, finally, let me tell you that the success of CBMTS-Industry I is our common success. We suppose that we all share the same thoughts to start preparing for the next CBMTS, which will take place in Dubrovnik in April 2001.

We would particularly like to say thank you to Richard and Barbara Price without whose energy and impulse as well as organization this symposium would not have been so successful as it was. On the base of very successful CBMTS-Industry I, we have already started organizational preparations for the next meeting with maybe the very ambitious title "The First World Congress of Chemical and Biological Terrorism".

Again, thank you for this opportunity to address you. On behalf of the Organizing Committee of CBMTS-Industry I, I wish you a lot of success at the symposium CBMTS-II.

16. OPERATIONAL ASPECTS OF CBMTS INDUSTRY I

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INTRODUCTION

CBMTS Industry I Symposium, Eco-Terrorism Chemical and Biological Warfare without Chemical and Biological Weapons, was the fifth in the series of CBMTS Symposiums, and it took place in Dubrovnik from 25-31 October 1998. The main organizers of the Symposium were ASA (Applied Science and Analysis Inc. from Portland, Maine) and Ministry of Defense of the Republic of Croatia.

Present at the Symposium were 125 scientist and medical professionals from 25 countries, while the demonstration, which took place on the 26th of October was attended by 165 professionals from 32 countries.

The goal of the Symposium was to point to the ever-growing danger from new ways and methods of warfare. That is, conventional and terrorist attacks on chemical, petrochemical, oil, pharmaceutical and other facilities, which in the production process use large quantities of toxic materials, as well as chemical and biological terrorism utilizing chemical and biological agents, and alternative chemical and biological means. The task was to point out the difficulties associated with prevention, protection, evacuation, rescue and medical treatment, and to come up with solutions.

The general opinion of the participants as well as of many other individuals, government and non-government institutions, and international organizations was that the Symposium was a complete success. My colleague Mr. Bokan elaborated the scientific and expert aspects of CBMTS Industry I.

As one of the organizers, as well as a professional soldier and chemist, I would like to say a few things regarding the exercises that were part of the Symposium.

THE EXERCISE IN KUTINA

"Absolutely unbelievable" or "unlike any one before" were just the two of many things heard from the participants as well as from OPCW representatives about the exercises and the message they carried. Why?

First, because the scenario of the exercise was not made up. It was based on real events, and as such, they were a real threat with scientifically proven consequences to people and environment not only in Croatia, but in neighboring countries, as well.

To make the exercise as real as possible, it took place near the mineral fertilizer factory that was attacked six times during the war. Among the participants of the exercises were civil protection forces, fire fighting units, military units from NBC and engineering, unit of technical protection and engineering from the factory, health services of the Republic of Croatia, citizens of Kutina, and the national television, which enabled the observers to see the complete exercise by transmitting it over the large screen.

Secondly, the exercises showed that intentional attacks or terrorist attacks with an intent to release toxic chemicals and cause chemical accidents, can cause the same, if not worse, consequences as if chemical weapons were used (Kuwait, Croatia).

Thirdly, the exercises pointed out how non-conventional warfare has similar results as chemical warfare. In this case, one's own chemical and biological facilities, warehouses and scientific institutions can become chemical and even biological weapons if they were to be targeted in the event of war or by terrorists. In this event conventional weapons of the party carrying out the attack become the initiators for the release of the toxic particles.

Fourthly, and maybe most importantly, as agreed by OPCW representatives, the exercises and the Symposium showed the possibility to bypass the Convention for Prohibition of Chemical Weapons. As a result this subject matter will most certainly be the topic of many future discussions.

I believe that the best argument for these statements is the film of the exercise. The film you are about to see is extracted from the original video of the exercise. (This film is available on CD version of CBMTS Industry II)

RESULTS

For the positive results of this exercise, the organizers received a number of verbal and written praises, messages of encouragement, proposals and offers from individuals, companies and scientific institutions to organize the next in the series of CBMTS Symposiums with the working title of CBMTS-Industry II, The First World Congress on Chemical and Biological Terrorism (science, medicine and anti-terrorism measures) Dubrovnik, Croatia, 22-27 April 2001.

With your help and cooperation the mentioned World Congress will be held in Dubrovnik with the following topics:

Defining Terrorism,
Preparation and Response,
Medical,
Medicine,
Identification,
Toxicology,
Psychological,
Communications,
Legal,
Intelligence,
Vulnerability Analyses,
Training,
Conference exercise(s) and sponsor participation

Your advice, proposals, critiques or help will be gladly accepted.

KEYWORDS
CBMTS Industry-I, Kutina, chemical response during war, chemical terrorism

17. ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE ACTIVITY MEASUREMENTS IN HUMAN BLOOD BY THE ELLMAN METHOD. I. EVALUATION OF PROCEDURE PROTOCOL

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INTRODUCTION

Absorption of organophosphorus compounds or carbamates is commonly assessed by measuring the decrease in acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) activities in human blood. The procedure for plasma BChE activities is well established while this is not the case for the erythrocyte AChE. We suggest a procedure based upon separation of plasma from erythrocytes followed by separate measurements of BChE in plasma and AChE in unwashed erythrocytes. The enzyme activities are measured by the spectrophotometric method of Ellman (1).

EXPERIMENTAL PROCEDURE AND CONDITIONS

Venous blood is collected into dried heparinized tubes and subjected to centrifugation. Plasma is then withdrawn. and it is essential to withdraw all plasma without removing any erythrocytes. The erythrocytes are then suspended in water in a volume corresponding to the initial volume of whole blood, followed by 60-fold dilution with buffer (0.1 M phosphate buffer pH=7.4) and then frozen in order to haemolyse the erythrocytes. After thawing, the suspension is further diluted with buffer and the thiol reagent DTNB added (final DTNB conc. during enzyme assay 0.33 mM). Ten minutes after DTNB, acetylthiocholine (ATCh) is added (final ATCh conc. during enzyme assay 1.0 mM) and the increase in absorbance read at 412 nm against a blank containing haemolysed erythrocytes suspended in buffer. The BChE activity in plasma is also measured with 1.0 mM ATCh using the same buffer and DTNB reagent. The final assay volume is 3.0 mL. During enzyme assay, the final dilution of erythrocytes is 600-fold (in relation to whole blood) and of plasma 150-fold. The enzyme activities are measured at 25 or 37 °C.

The concentration of haemoglobin in the erythrocyte suspension is determined spectrophotometrically with a modified Zijlstra reagent containing potassium ferricyanide, sodium bicarbonate and Triton X-100 (2, 3). The final reaction volume is 1.5 mL, and the final erythrocyte dilution 120-fold. The absorbance is read against buffer at 546 nm at room temperature.

The activities of AChE are expressed as micromoles hydrolysed ATCh per minute and per milliliter whole blood, or per milligram haemoglobin. The activities of BChE are expressed per milliliter of plasma.

DISCUSSION

The described experimental conditions were evaluated in a previous study concerning routine measurements of cholinesterase activities in human whole blood and plasma (4). Phosphate buffer was chosen because the effect of temperature on the pH of that buffer is small, and the buffer can therefore be prepared and used over a broad temperature range (10 to 40 °C). ATCh is a suitable substrate for AChE and BChE. For routine purposes it is convenient to use the same substrate concentration (1.0 mM) for both enzymes. At pH=7.4 the rate of spontaneous hydrolysis of 1.0 mM ATCh is slow and corrections for the non-enzymic substrate hydrolysis are required only at 37 °C.

The absorbance of haemolysed unwashed erythrocytes is not stable. The absorbance measured against buffer decreases about 0.006 absorbance units over 10 min at 25 or 37 °C. That decrease is very small and need not be taken into account particularly because it is suggested that activities are measured against suspended haemolysed unwashed erythrocytes as a blank.

DTNB reacts not only with thiocholine, but also with thiol groups in the haemolysed erythrocytes. Under the above experimental conditions the reaction of DTNB with thiol groups in the haemolysed erythrocytes is completed within 10 min, and for that reason it is suggested to add the substrate 10 min after addition of DTNB. The reaction of DTNB with thiol groups in the erythrocytes reduces the DTNB conc. by only about 0.5%.

The suggested procedure is well reproducible. The within-run and between-run imprecision for the activity measurements of AChE in unwashed haemolysed erythrocytes are 2% each, and for the haemoglobin determination 1% each. The corresponding imprecision for plasma BChE activity measurements are 1-3 and 6-7% respectively (this paper and ref. 5).

SUMMARY

For routine assay of human blood cholinesterase activities it is suggested to separate erythrocytes from plasma by centrifugation and measure the acetylcholinesterase activity in unwashed erythrocytes and butyrylcholinesterase activity in plasma. The suggested substrate for both enzyme activity measurements is 1.0 mM acetylthiocholine.

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KEYWORDS

Acetylcholinesterase, butyrylcholinesterase, activity measurement, human plasma, human erythrocytes

18. ORGANOPHOSPHORUS COMPOUNDS AND ESTERASES: CURRENT RESEARCH TOPICS CONCERNING TOXICITY OF, AND PROTECTION AGAINST ORGANOPHOSPHATES

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INTRODUCTION

Two groups of esterases react with organophosphorus (OP) compounds: serine esterases and phosphoric triester hydrolases (PTHs; EC 3.8.1). The acute toxicity of OP compounds is primarily due to inhibition of acetylcholinesterase (AChE; EC 3.1.1.7). The delayed polyneuropathy, induced by some OP compounds, is related to inhibition of the neuropathy target esterase (NTE). So far, NTE has not been classified in the Enzyme Nomenclature (1). AChE and NTE are both serine esterases. Unlike to serine esterases, OP compounds are not inhibitors but substrates of phosphoric triester hydrolases (PTHs): paraoxonase (PON; EC 3.1.8.1) and DFPase (EC 3.1.8.2). The hydrolysis products of the OP compounds are not toxic, and PTHs therefore play an important role in OP detoxification.

The structural formulae of several OP compounds are given in Figure 1. Sarin, soman, tabun, and VX are nerve agents. Paraoxon and DFP are characteristic substrates of the PTHs. Both serine esterases and PTHs react with OP compounds on the same ester or anhydride bond marked in the figure by an undulated line.

For detailed information, readers are invited to consult books and conference proceedings listed in references 2-13 at the end of this brief review. Reference 14 summarizes the early literature on the subject. Individual papers are not quoted in the text, because their number would grossly exceed the length of the review.

REACTIONS OF ORGANOPHOSPHORUS COMPOUNDS WITH SERINE ESTERASES

The reactions of serine esterases with OP compounds are shown in Scheme 1. Inhibition of serine esterases is due to phosphorylation of the active-site serine. This reaction proceeds via an intermediate Michaelis-type complex between the enzyme and OP compound. The phosphorylated enzyme (EP) is catalytically inactive. Dephosphorylation with water (spontaneous reactivation) is very slow, while it is faster with oximes (oxime reactivation). Depending on the substituents on the phosphorus, EP can undergo dealkylation, which is termed ageing. The aged enzyme cannot be reactivated.

AChE is a globular protein and its three-dimensional structure is known. Its physiological substrate is acetylcholine. A schematic drawing of the AChE molecule is shown in Figure 2. The active site of AChE is in the center of the molecule accessible through a narrow gorge lined with water molecules. The catalytic triad (serine, histidine and glutamic acid), a choline-binding pocket and an acyl-binding pocket form the active site. Furthermore, AChE has an allosteric (peripheral) site close to the rim of the gorge. The allosteric site is catalytically inactive. However, reversible binding of substrates or other ligands to that site affects catalysis in the active site. The effect is usually inhibitory. There is some evidence that the AChE molecule might have a "back door" through which products of substrate hydrolysis leave the enzyme.

Research on AChE is now directed towards identification of individual amino acids that participate in the individual steps of catalysis. The experimental approach consists in preparing site-directed mutants, correlating the catalytic properties with the structure of the mutant and modeling the enzyme/ligand complexes. The results are expected to further elucidate the mechanism of action of AChE. This in turn might facilitate a more rational design of compounds, which would prevent phosphorylation of the active site by OPs, facilitate reactivation of EP and slow down aging. The toxicity of OPs is primarily determined by these three reactions.

NTE has only recently been cloned and was shown to be unrelated to any known serine hydrolases. Its physiological role is not known. OP compounds are inhibitors of NTE, but only some OPs cause delayed polyneuropathy. These OPs phosphorylate the active-site serine, which in turn is followed by aging of the inhibited NTE. Polyneuropathy does not develop if ageing does not occur. This could indicate that the axonal maintenance is sensitive to the negative charge on the aged enzyme (Scheme 1) and not to the activity of NTE. Phosphorylation and aging of NTE occur within minutes, but degeneration of the long axons takes weeks to develop.

Present studies aim at clarifying the link between NTE and the OP-induced delayed polyneuropathy. Studies are focused on the molecular structure of NTE and on compounds that promote OP-induced polyneuropathy without causing polyneuropathy themselves. Promoters known so far are NTE inhibitors, but it seems unlikely that NTE is

the target enzyme.

HYDROLYSIS OF ORGANOPHOSPHORUS COMPOUNDS BY PHOSPHORIC TRIESTER HYDROLASES

The mechanism of reaction of OP compounds with PTHs is different from the reaction of OPs with serine esterases. PTHs require divalent cations for catalysis as shown in Scheme 2. The cation (M^{++} in Scheme 2) is embedded in histidine residues and it binds the water molecule required for OP hydrolysis. So far nothing is known about the intermediate steps leading to hydrolysis, except for the kinetic evidence that a Michaelis complex is formed between the enzyme and OP. The physiological role of PTHs is also not known.

PTHs hydrolyse a broad range of OP compounds. The substrate specificities of paraoxonase and DFPase are different, and the same holds for the cation required for catalysis. Paraoxonase in mammalian sera requires calcium ions, while DFPase in microorganisms usually depends on magnesium, zinc or manganese cations. Recently DFPase was also found in plants and its activity was stimulated by manganese ions. PTHs are stereoselective like AChE. The OP enantiomer, which is more quickly hydrolysed by PTHs, is less inhibitory for AChE, and vice versa. PTHs have been shown to hydrolyse carboxylic acid esters such as phenylacetate, which is a characteristic substrate of the arylesterase (EC 3.1.1.2) (1).

The search for the natural substrate(s) and physiological role of PTHs continues. Mammalian serum paraoxonases seem to act against cellular damage from toxic agents and oxidized lipids, and there are some indications that human serum paraoxonases might be markers of lipid metabolism disorders. The polymorphism of these enzymes in human sera is well established. PTHs have recently been cloned and mutants prepared. This has greatly enhanced studies on substrate specificity and mechanism of substrate hydrolysis. The in vivo role of PTHs in detoxification of OPs and their use in decontamination from OPs constitutes a very lively field of research.

OXIMES AS ANTIDOTES AND PROTECTORS AGAINST ORGANOPHOSPHORUS COMPOUNDS

The primary mechanism of action of oximes as antidotes is to reactivate the phosphorylated AChE (Scheme 1). The pyridinium oximes PAM-2 and HI-6 (Figure 2), and the bis-pyridinium dioxime toxogononin, are therapeutic drugs in OP poisoning. Not a single oxime prepared so far is active against a very broad range of OPs. Moreover, none has been known to act against all four nerve agents. The search for better antidotes is still based on the trial and error approach. The same applies to oximes and other compounds used as prophylactic agents. Imidazolium oximes, and more recently quinuclidinium derivatives, are under evaluation; two compounds from those groups are shown in Figure 2.

The products of dephosphorylation by oximes are phosphorylated oximes (Scheme 1). Phosphorylated oximes are potent AChE inhibitors. Many, but not all, are very unstable compounds. In vivo phosphorylation of the reactivated AChE by phosphorylated oximes has seldom been reported so far. Oximes themselves are also toxic. They bind to AChE as reversible inhibitors.

Reversible inhibitors form complexes with AChE either in the active site or in the allosteric site or in both sites of the enzyme. Reversible inhibitors, including oximes, protect thereby AChE from phosphorylation. When the reversible inhibitor binds to the active site, the protection is due to direct competition between the OP compound and reversible inhibitor. Binding of a reversible inhibitor to the allosteric site induces indirect protection of the active site. This has been well documented by in vitro studies, but was so far less evaluated in experimental toxicology.

ESTERASES AS ANTIDOTES, PROTECTORS AND DECONTAMINATING AGENTS AGAINST ORGANOPHOSPHORUS COMPOUNDS

Phosphorylation of serine esterases by OP compounds occurs on a 1:1 molar basis. This reaction inhibits the enzyme, but detoxifies the OP compound. Phosphorylation of AChE causes toxicity, because phosphorylated AChE cannot hydrolyse its physiological substrate acetylcholine. On the other hand, butyrylcholinesterase (BChE; EC 3.1.1.8) and carboxylesterase (EC 3.1.1.1) in mammalian sera and tissues can be almost completely inhibited without an apparent toxic effect. This means that cholinesterases and carboxylesterases act as scavengers of OPs. However, once phosphorylated, the enzymes cannot detoxify another OP molecule unless oximes are present which reactivate the enzyme. Consequently, cholinesterases and carboxylesterases act as stoichiometric scavengers, while combined with oximes they become catalytic scavengers.

The feasibility of using serine esterases, such as AChE and BChE combined with oximes, as drugs against OPs has been demonstrated in rodents and in non-human primates. Protein engineering techniques have now enabled an intensive search for enzyme mutants, which ideally should meet the requirement to react rapidly with OPs, to age

slowly and to be easily reactivatable by oximes.

PTHs also play a major role in the detoxification of OPs. The high toxicity of OPs for avian species has been attributed to the low activity of endogenous PTHs in these species. Rabbits, which have a very high paraoxonase activity, are more resistant to paraoxon than other mammals. Human serum paraoxonases exhibit a substrate dependent polymorphism, with low and high activity modes for each substrate. Analysis of polymorphisms in population groups is suggested to identify individuals at risk. Studies on rodents have shown that administration of purified paraoxonase significantly reduces the toxicity of paraoxon and other OPs. These promising results stimulate present research on the use of PTHs and their mutants as drugs against OPs.

Finally, the use of esterases is suggested for the decontamination of skin, clothing and equipment. PTHs from a microorganism immobilized to cotton wipes detoxifies nerve agents and OP pesticides. Purified AChE or BChE immobilized to polyurethane sponge does the same. Rinsing the sponge with oxime solutions restores the enzyme activity. PTHs can be co-immobilized on the same sponge thus increasing the decontaminating capacity. Enzymes immobilized to matrices are more stable than in solution and can therefore be used repeatedly.

SUMMARY

Reactions of organophosphorus compounds with cholinesterases, neuropathy target esterase and phosphoric triester hydrolases are discussed with respect to the toxicity of organophosphates. Antidotes, protectors and decontaminating agents against organophosphates are described.

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KEYWORDS

Serine esterases; Cholinesterases; Acetylcholinesterase; Butyrylcholinesterase; Neuropathy target esterase; Phosphoric triester hydrolases; Paraoxonase; DFPase; Oximes

FIGURES AND TABLES

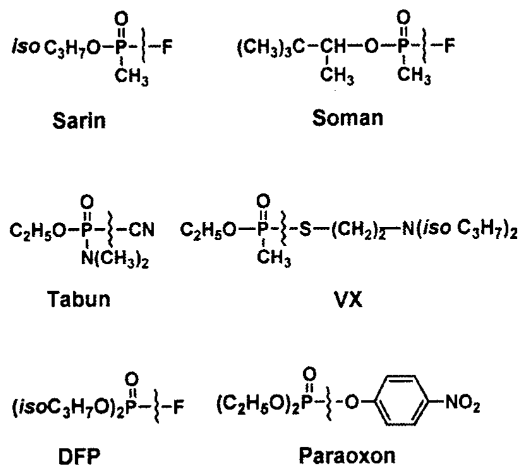


Figure 2. Schematic drawing of the AChE molecule (prepared by Zoran Radic, UCSD, USA)

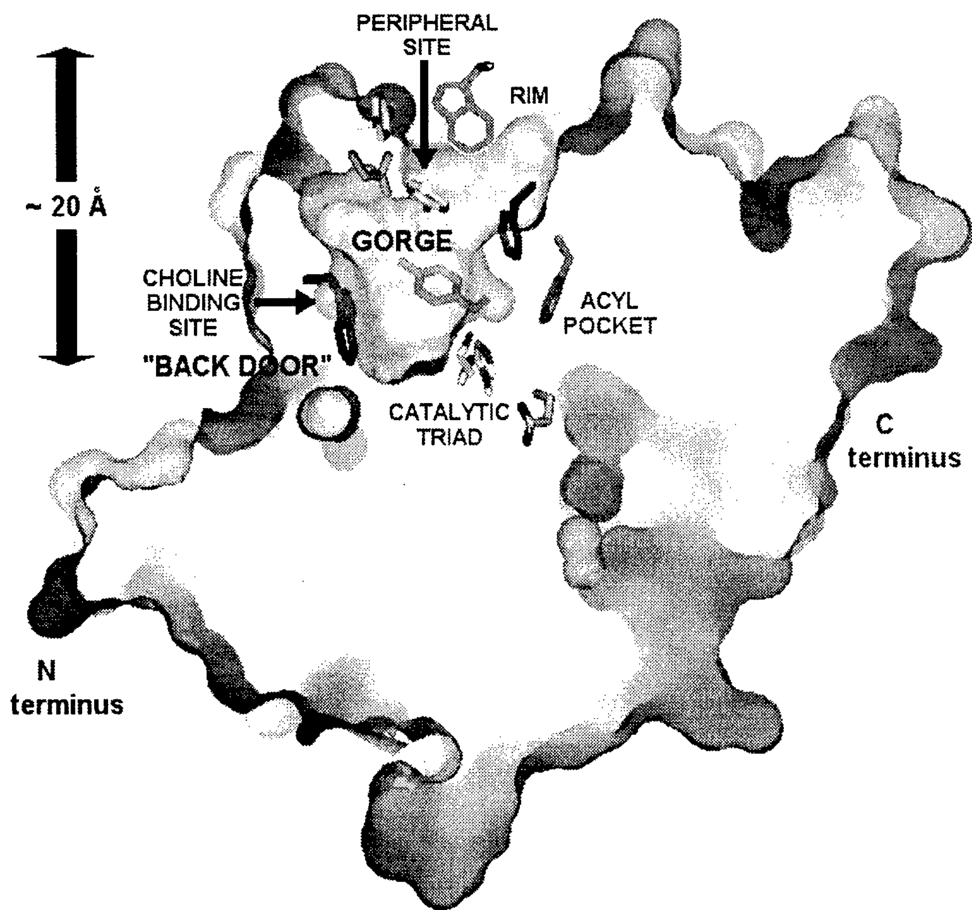
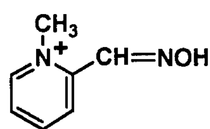
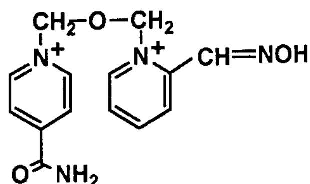


Figure 3. Structural formulae of several oximes

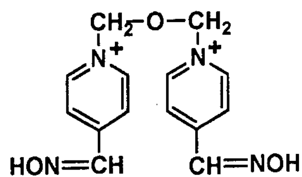
Pyridinium oximes



PAM-2

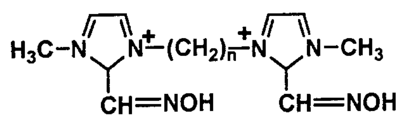


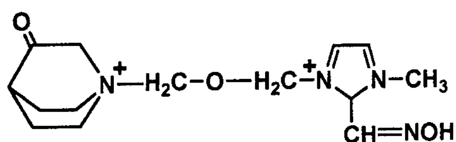
HI-6



Toxogonin

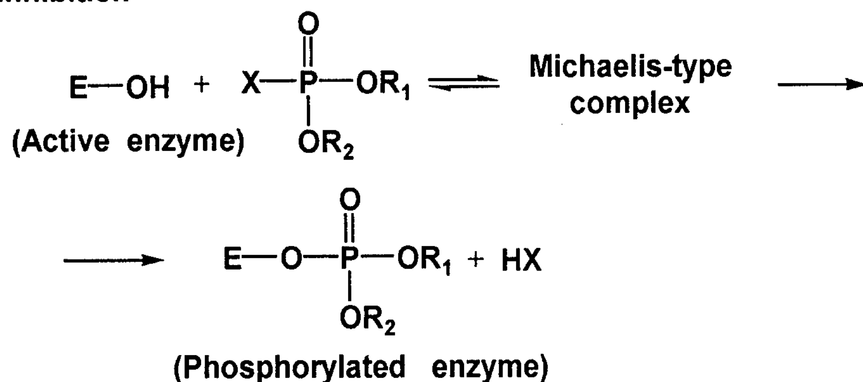
Imidazolium oximes



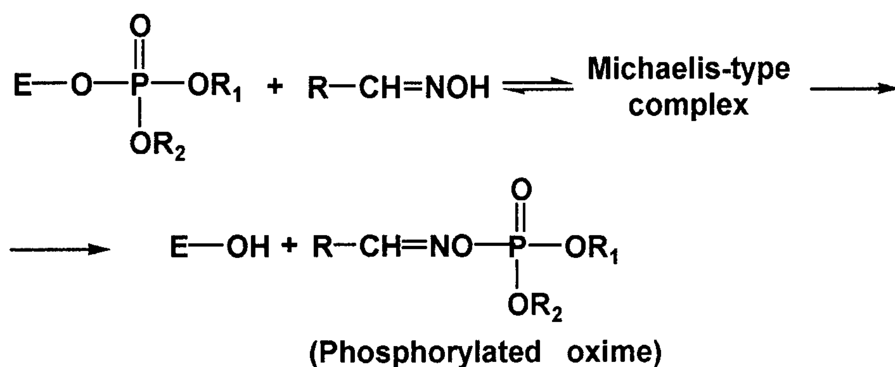


Scheme 1. Reactions of serine esterases with organophosphorus compounds

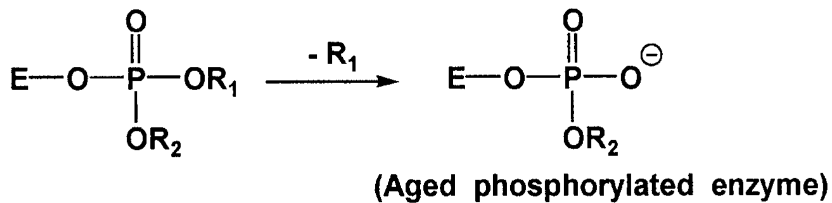
Inhibition



Oxime reactivation



Ageing (dealkylation)



Scheme 2. Reaction of phosphoric triester hydrolases with organophosphorus compounds

$$E\text{-}M^{++} + H_2O + X\text{—}P(=O)(OR_1)(OR_2) \rightleftharpoons \text{Michaelis-type complex} \rightarrow$$

$$\rightarrow E\text{-}M^{++} + HX + HO\text{—}P(=O)(OR_1)(OR_2)$$

19. ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE ACTIVITY MEASUREMENTS IN HUMAN BLOOD BY THE ELLMAN METHOD. II. COMPARISON OF THE PROCEDURE PROTOCOL WITH PROCEDURES SUGGESTED BY OTHER AUTHORS

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INTRODUCTION

Erythrocyte acetylcholinesterase (AChE; EC 3.1.1.7) activities are usually measured in whole blood and the most commonly used substrate is acetylthiocholine (ATCh). As whole blood contains also butyrylcholinesterase (BChE; EC 3.1.1.8) the rate of substrate hydrolysis in whole blood is due to the activity of both enzymes.

Several procedures have been proposed so far for erythrocyte AChE measurements in whole blood. Worek et al. (1) propose to measure AChE activity in the presence of 20 μM ethopropazine which inhibits plasma BChE. Portmann (2) and Wicki (3) propose to measure both AChE and BChE in whole blood by successive addition of ATCh and butyrylthiocholine (BTCh). An assessment of erythrocyte AChE activity can also be done by measuring the rate of hydrolysis of 1.0 mM ATCh in whole blood, as under these conditions the rate of hydrolysis is primarily due to the erythrocyte AChE (4, 5). Finally, one can separate by centrifugation the erythrocytes from plasma, wash the erythrocytes with saline or buffer, and measure AChE activities in washed erythrocytes and BChE in plasma. In our procedure described in Part I of this paper we also suggest to separate erythrocytes from plasma, but we suggest to measure AChE in unwashed erythrocytes.

We have compared the protocols described above and assessed advantages and disadvantages of each procedure.

RESULTS

Unwashed erythrocytes contain some residual plasma. In order to test whether BChE in the residual plasma affects the AChE activity in unwashed erythrocytes, we applied ethopropazine as a potent BChE inhibitor. The mean rate of hydrolysis of 1.0 or 0.5 mM ATCh was 8% inhibited by 20 μM ethopropazine (4 samples) irrespective of whether the activity was measured in unwashed or washed erythrocytes. This result indicates that BChE in residual plasma did not show up in the assay, as otherwise the inhibition in unwashed erythrocytes would be higher than in washed erythrocytes. We therefore consider that washing the erythrocytes before AChE assay is not required, particularly because washing might cause haemolysis, and also some detachment of AChE from the membrane. However, the above result indicates that the 8% inhibition must be attributed to inhibition of AChE, which is in agreement with 5% inhibition of AChE reported by Worek et al.(1).

A further test concerning the procedure was the following. The cholinesterase activity of whole blood was measured with 1.0 mM ATCh without separating erythrocytes from plasma. Knowing the haematocrit, the cholinesterase activity of the whole blood sample was calculated as the sum of separate measurements of AChE in unwashed erythrocytes and BChE in plasma. The mean values of the measured and calculated activities were in very good agreement: 7.16 and 6.97 $\mu mol\ min^{-1} mL^{-1}$ respectively (15 samples).

The AChE activities can be expressed either per volume of whole blood or per haemoglobin content. The relative standard deviation of the mean erythrocyte AChE activities in 15 samples was 12% irrespective of whether the activity was expressed per milliliter blood or milligram haemoglobin. The mean haemoglobin concentration in 15 samples of whole blood and unwashed erythrocytes was 8.82 and 8.28 μmol/mL respectively. This difference is small, but it might point to a slight loss of haemoglobin due centrifugation and plasma withdrawal.

DISCUSSION AND CONCLUSIONS

To detect absorption of organophosphorus compounds or carbamates one should measure the activity of both cholinesterases in human blood, because inhibition of AChE and BChE by a given compound is different and there is no general rule to predict which enzyme will be more inhibited.

We consider that the procedure outlined in Part I is simple and therefore suitable for routine assays, particularly because we suggest to use only one substrate (ATCh) and measure both enzymes with the same substrate concentration. The procedure however requires centrifugation of whole blood and a careful separation of plasma from erythrocytes, and this might be a disadvantage.

Worek et al. (1) suggest an AChE assay in whole blood with ATCh as substrate, but in the presence of ethopropazine (20 μM) in order to inhibit BChE. When ethopropazine is applied as a selective inhibitor of plasma

BChE one should bear in mind that the usual BChE phenotype is 98% inhibited with 20 μM ethopropazine, while the atypical BChE phenotype is inhibited only 74%, and the heterozygots of the atypical variant between 94 and 85% (6). The occurrence of BChE phenotypes other than the usual is rare (7, 8) and the errors in estimated AChE activities due to their presence should therefore be rare. Worek et al. (1) further suggest that plasma BChE is measured with BTCh as substrate. Consequently, determination of AChE and BChE requires two different substrates, one selective inhibitor and centrifugation of whole blood to obtain the plasma.

In the procedure of Portmann (2) and Wicki (3) AChE and BChE are both measured in whole blood. The activity of both enzymes is first measured with ATCh (1.0 mM) after which BTCh (5.0 mM) is added to the same cuvette and the activity measurement continued. Assuming that BTCh is hydrolysed only by BChE and that addition of BTCh stops the hydrolysis of ATCh, the activities of AChE and BChE are calculated. This protocol avoids any blood centrifugation which is an advantage. In that procedure one should however bear in mind that BTCh is not fully specific for BChE, but is also hydrolysed by AChE. At the above substrate concentrations AChE hydrolyses BTCh about 10-times slower than ATCh (mean of 5 samples).

In conclusion, each procedure described above has its own intrinsic problems. The protocol of choice should be the simplest procedure in technical terms, giving reliable and well reproducible results. That should be verified in a quality control study over a wide range of AChE and BChE activities in order to determine which protocol is most selective in the differentiation of the two enzymes' activities.

SUMMARY

The procedure suggested for the assay of erythrocyte acetylcholinesterase and plasma butyrylcholinesterase in human blood outlined in Part I of this paper is compared with two other procedures suggested for these two enzymes. Advantages and disadvantages of each protocol are discussed.

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KEYWORDS

Acetylcholinesterase, butyrylcholinesterase, activity measurement, human plasma, human erythrocytes

20. EFFECT OF PYRIDOSTIGMINE (PYR) AND NON PENETRATIVE TRAUMATIC BRAIN INJURY ON NEUROMUSCULAR PERFORMANCE IN THE RAT

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INTRODUCTION

Pyridostigmine, a reversible carbamate acetylchlorinesterase, has been recommended as a pretreatment in warfare nerve agent intoxification. It is used by the military to obtain 20-30% whole blood acetylcholinesterase (AchE) inhibition to enhance the effectiveness of the standard therapeutic regimen jase (ChE) inhibitions, when administered in symptom producing doses, may cause rhabdomyonecrosis [3]. Morphological alterations of the neuromuscular junction have been demonstrated in diaphragm, soleus and extensor digitorum longus muscles of rats given PYR either acutely or subacutely [4-6]. There have been relatively few studies documenting the functional effects of PYR on the neuromuscular junction. Whether PYR exert an adverse on neuromuscular activities with closed head injury has not been documented.

Closed head injury is associated with high mortality and morbidity. It has been reported that survivors of closed head injury may develop cognitive dysfunctions and retarded neuromuscular performance. Whether it may exert an adverse effect on neuromuscular activities in the survivors with closed head injury has not been studied.

The present study was designed to investigate the effect of PYR on neuromuscular performance in rats subjected to closed head injury [7].

MATERIALS AND METHODS

Male Sprague rats (300+5g) were pretreated with normal saline or PYR (7 mg/kg body weight, intraperitoneally), daily for 3 days. They were subjected to (a) rotametric strength test (b) forelimb grip test, 3 h after the last injection. They were anesthetized with a Clinical Research Center cocktail (0.3 ml per 100 g body weight) and were subjected to a closed head injury of weight 300 g height 1.5m. They were subjected to the rotametric test and grip strength tests after 16 or 40 h.

Rotametric test - A rotametric device (Columbus Instruments Rotamex 4/8 system. Ohio, USA) was used to examine the ability of the animal to coordinate itself while being placed on a rotating rod with the rotational speed of 6 rpm (start speed) and 20 rpm (end speed) for a period of 120 seconds. An internal micro-controller was used to detect the time when a subject fell from the rod. The average reading (in seconds) for two successive trials was taken from each animal.

Grip-strength test - The fore limb grip-strength was measured using a grip-strength meter (Columbus Instruments, Ohio, USA). The animal was placed on the electronic digital force gauge that measures the peak force exerted on it by the action of the animal. While drawing along a straight line leading away from the sensor, the animal was released at some point and the maximum force attained was stored on the display. The highest reading (in Newton) of three successive trials was taken from each animal.

Induction of head injury - The scalp of the anesthetized animal was shaved, and a helmet was cemented to the calvaria with a thin layer of dental acrylic. The animal was then placed in a prone position on a foam bed, with the lower end of the Plexiglas tube positioned directly above the helmet. The injury was delivered by dropping 300 g of weight from 1.5 m.

The weight-drop device consists of a column of brass weights falling freely by gravity onto a metallic helmet fixed to the skull vertex of the rat by dental acrylic. The brass weights, each about 50 g, are threaded so that they can be connected to produce a falling weight ranging from 50 to 500 g. From a designated height, the weight falls through the vertical section of a transparent Plexiglas tube held in place with a ring stand. The helmet consists of a stainless steel disc, 10 mm in diameter and 3 mm thick. The contact side of the disc is grooved concentrically to accept acrylic and firm the contact. After release of the weight, the Plexiglas frame is removed rapidly to prevent a second impact.

RESULTS AND DISCUSSION

Rotametric score - Saline-pretreated rats were associated with a rotametric score of 114$\pm$4 sec. With sham operation. the score was 117$\pm$3 and >120 seconds at 16 and 40 h, respectively. When subjected to closed head injury, the score was 98$\pm$13 and > 120 sec at 16 and 40 h, respectively. Pyridostigmine pre-treatment, however, reduced thee rotametric score to 94$\pm$8. With sham-operation, the rotametric score was 83$\pm$2 and 87$\pm$2 at 16 and 40 h. respectively. With closed head injury, the rotametric score was 105$\pm$12 and 119$\pm$1 at 16 and 40 h, respectively.

Fore limb grip-strength score - Saline pre-treated rats obtained a grip-strength score of 8.3±0.3. With sham operation, the grip-strength score was 8.2±0.7 and 7.7±0.9 at 16 and 40 h, respectively. In contrast, close head injury reduced the grip-strength score to 6.9±0.5 and 7.3±1.0 at 16 and 40 h, respectively. Pyridostigmine administration was associated with a grip-strength score of 6.3±0.3 ($p<0.05$ vs. saline-treated group). With sham-operation, the grip-strength score was 6.2±1.1 and 7.3±0.9 at 16 and 40 h, respectively. With closed head injury, the score was 5.8±0.6 and 6.8±1.9 at 16 and 40 h, respectively.

The results showed that PYR administration caused a significant decrease in rotametric and forelimb grip-strength scores in rats. However, no statistically significant decrement in performances could be evidence after 16 and 40 h. These observations suggested that PYR may have a short term deleterious effect on the neuromuscular performance in rats. Closed head injury caused a numerical decrease in the rotametric and grip-strength scores, but statistically significant decrement was not observed. The results showed that there was no synergistic effect between PYR administration and closed head injury on neuromuscular performance in rats. The significance of these observations remains to be clarified and the present findings need to be substantiated in humans.

SUMMARY

The effect of pyridostigmine (PYR) and closed head injury on neuromuscular performance in the rat is described. The rats were pretreated with either saline or PYR (7 mg/kg body weight, IP) daily for 3 days before being subjected to rotametric and grip-strength tests. They were then subjected to closed head injury and underwent the tests. PYR administration caused a significant short-term decrement in rotametric and grip-strength performances suggesting that PYR may exert a short-term deleterious effect on the neuromuscular performance in the rat. Closed head injury caused a numerical decrease in the rotametric and grip-strength scores but the effect was not statistically significant. There was no synergistic effect between PYR and closed head injury on the neuromuscular performance in the rat.

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KEYWORDS

Rotametry, grip-strength, acetylcholinesterase inhibition

FIGURES

Figure 1. Effect of pyridostigmine and closed head injury on rotametric performance in the rat.

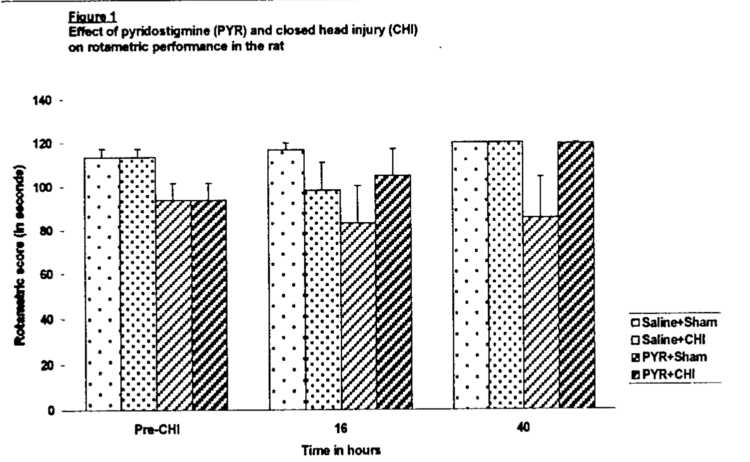
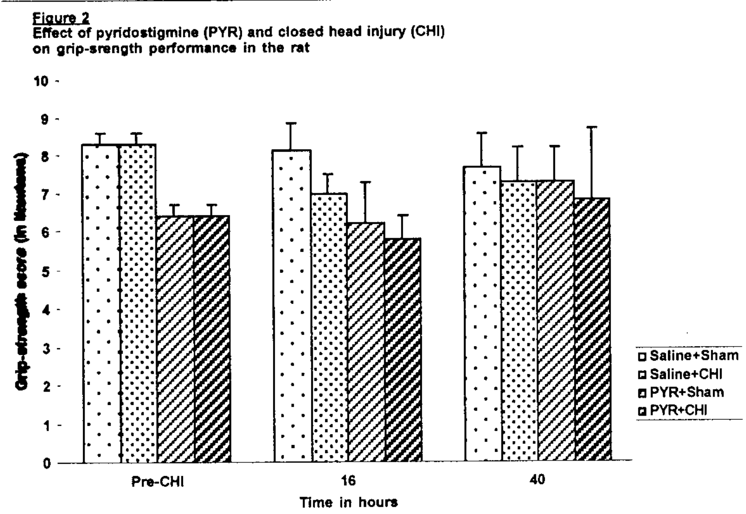


Figure 2. Effect of pyridostigmine and closed head injury on grip-strength performance in the rat.



21. THE RELATIONSHIP BETWEEN THE DOSE OF SARIN AND CHOLINESTERASE INHIBITION IN THE BLOOD FOLLOWING INHALATION INTOXICATION

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SUMMARY

Inhalation exposure is one of the important routes of administration of nerve agents. There are scarce data dealing with the effect of low concentrations of these agents. We studied effects of sublethal concentrations of sarin administered by inhalation on acetylcholinesterase (AChE, EC 3.1.1.7) activity in the blood of rats. The aim of this study was to determine if it is possible to monitor exposures to low concentrations of sarin using current methods of determining blood cholinesterases. We used a dynamic inhalation chamber of our own construction. AChE was determined according to modified Ellman's method. The concentrations of sarin were the following: 0.5, 0.67, 0.8 and 1.0 $mg.m^{-3}$, 4 hrs exposure. At the concentration of 0.5 $mg.m^{-3}$, the effects of duration of exposure were studied in intervals 3, 4 and 6 hrs, respectively. Different concentrations of sarin (4 hrs exposure) caused decreases of AChE activity from 71.9% (the lowest concentration of sarin) to 40.4% (the highest concentration of sarin). Increased duration of exposure has led to increased AChE inhibition, dependent on the time of exposure. At the last interval studied (6 hrs), AChE activity was 60%. It appears from these results that AChE in the blood is very sensitive marker for diagnosis of inhalation exposure to low concentrations of sarin.

INTRODUCTION

Intoxication with organophosphorus cholinesterase inhibitors constitutes a problem in industrial and agriculture toxicology. Similar compounds, e.g., sarin are considered as potential chemical warfare agents due to their toxicity and physicochemical properties. The main route of toxic compounds to the body is through the respiratory or gastrointestinal tract and the skin. A large number of results concerning parenteral administration has been published (1), however, few experiments have been published on inhalation intoxication, especially for very low concentrations (2-4).

MATERIAL AND METHODS

Animals

Female Wistar rats (VELAZ Prague), weighing 180-200 g, were used. The animals were maintained in an air-conditioned room (22°C and 50% relative humidity, with light from 7:00 to 19:00 h, and were allowed free access to standard chow and tap water. The Ethics Committee of Charles University at Hradec Králové supervised the handling of experimental animals.

Inhalation intoxication

A dynamic inhalation chamber of our own construction was used. It was composed from the chamber (total volume 30 L) with active exchange of air (30 L/min) with suction of air through the filter, i.e., the chamber of dynamic character was used. The formation of sarin vapors was based on evaporation of sarin in a rotating bar. The concentration of sarin in the chamber was measured using detection equipment based on determination of cholinesterase inhibition in the alcoholic extract of air and calibrated for sarin. This allowed us to monitor sarin concentration discontinuously. Concentrations of sarin were as follows: 0.5, 0.67, 0.8 and 1.0 mg m^{-3} for 4 hrs exposure. At the concentration of 0.5 mg m^{-3}, the effects of exposure duration were studied in intervals of 3, 4 and 6 hrs, respectively.

AChE activity determination

AChE activity of erythroctyes was determined with the modified method of Ellman et al., (5) as described elsewhere. Acetylthiocholine was used as substrate and activity was expressed as (cat/L of the whole blood).

Statistical evaluation

Statistical evaluation was made using the method of linear regression analysis calculated by means of minimal squares. The equations with relevant correlation coefficients were calculated. For each concentration and interval, six animals in each group were used.

RESULTS AND DISCUSSION

Exposure to different sarin concentrations caused decreases in AChE activity from 71.9% (concentration 0.5 mg m^{-3}) to 40.4% (the highest concentration). The relationship between AChE activity and concentration of sarin is shown in Table 1. At the highest concentration of sarin, with AChE activity at 40.4% of the uninhibited AChE, marked clinical symptoms of intoxication were not observed. AChE activity is dependent on the duration of exposure as it is demonstrated for concentration 0.5 mg m^{-3} in Table 2.

In all cases, both relationships (i.e., sarin concentration vs. AChE activity and duration of exposure vs AChE activity) were linear. These results suggest that at the low concentrations, AChE in the blood is "titrated" by sarin and that AChE in the blood is very sensitive marker for diagnosis of inhalation exposure to low concentrations of sarin.

ACKNOWLEDGEMENT

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KEY WORDS

sarin, inhalation, acetylcholinesterase, rat, blood

TABLES AND FIGURES

Table 1. Dependence of sarin concentration and AChE activity in the blood

Sarin Concentration (mg m^{-3})	AChE activity (% of controls, mean±SD)
0	100±12.1
0.5	71.9±13.1
0.67	61.4±10.2
0.8	50.7±9.1
1.0	40.4±9.2

Equation: $y = 100 - 59.1x$, $r_{xy} = -0.99844$

Table 2. Relationship between duration of sarin exposure and AChE activity in the blood

Exposure Time (hrs)	AChE activity (% of controls, mean±SD)
0	100±12.1
3	78.2±10.2
4	71.9±13.1
6	60.0±10.2

Equation: $y = 100 - 7.33x$, $r_{xy} = -0.99842$

22. SIMPLE METHOD FOR MORE PRECISE DIAGNOSIS AND TREATMENT OF NERVE AGENT POISONING

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INTRODUCTION

In the treatment of nerve agents (NA) poisoning, two therapeutic principles are used. Paralysis of accumulated acetylcholine is due through the action of anticholinergic drugs, most frequently atropine, cholinesterase reactivators are used for so called causal therapy (1,2). The stabile phosphorylated enzyme is possible to return to normal activity by these reactivators (3,4), under condition that the inhibited cholinsterase is not converted to non-reactivatable complex. It is known that NA produced this so called dealkylation or aging at different times after the some reaction of enzyme with inhibitor. For soman, the half time for dealkylation was determined to be about 5-10 min., for sarin about 10-15 hours and for VX the dealkylation was not possible to determine more than 24 hours (5).

These findings can be applied for the both cholinesterases (acetylcholinesterase, AChE, EC 3.1.1.7. and butyrylcholinesterase, BuChE, EC 3.1.1.8.) in the nervous system and in the blood. Knowledge of state of AChE (reactivatable of unreactivatable complex) would of interest for diagnosis, treatment and prognosis of NA poisoning.

Description of simple test for these purposes is the aim of this study.

MATERIALS AND METHODS

In experiments in vitro, the blood from 55 years-old healthy man was haemolyzed by distilled water (1:20) or by solutions of soman, sarin and VX compound, respectively. The concentrations of inhibitors caused high (80-90 %), middle (50 %) or low (10-20 %) inhibition. Following 30 min., 5 hours or 24 hours, AChE activity in these samples was deremined in the presence or the absence of trimedoxime (5.10^{-4} M).

In experiments in vivo, the dogs of both sexes, weighing 6,75 - 14 kg, were used. The animals were i.m. intoxicated with soman (0,007 mg/kg), sarin (0,03 mg/kg) or VX compound (0,003 mg/kg), respectively. The AChE activity and reactivation efficacy in the blood samples was determined at different time intervals after the intoxication. AChE activity was expressed as _mol of substrate hydrolyzed per ml of the blood per min, or as per cent of controls, the reactivation potency was expressed as per cent of reactivation.

The homogeneity of both control and experimental groups was evaluated by Bartletts test. For these experiments, 5-9 measurements were used for experiments in vitro (each time interval and every concentration) and 3-6 animals for experiments in vivo. The differences beween groups were determined by analysis of variance, using Hewlett Packard computer 9830 A.

RESULTS

Very low reactivation was demonstrated for soman inhibited AChE. AChE reactivation from 15 to 63 % for the blood AChE inhibited by sarin was observed and AChE reactivation higher than 55 % was determined for AChE inhibited by VX.

Following intoxication with soman, fast decrease of the blood AChE activity ws determined. It reaches to steady state 10 min after the injection of soman. The reactivation ability of the blood AChE was decreased in time and following 50 min the blood AChE in soman intoxicated dogs was not able to reactivate. The mean reactivatability in the range of 0 - 16 % for soman intoxication was observed.

In case of sarin, similar decrease of the blood AChE activity was demonstrated. On the other hand, reactivatability 100 min after the injection of sarin was observed, the mean reactivation for sarin inhibited AChE was determined to be approximately 60 %.

The prolonged AChE inhibition was demonstrated following VX intoxication: the steady state was achieved 30min after the injection of VX. On the other hand, practically 100 % reactivation in vivo was obtained 120 min after intoxication with VX. The results in vitro and in vivo are summarized in Table I.

Reactivation (%) of the human blood AChE in vitro and the dog AChE in vivo following exposition by sarin, soman and VX

Compound	% of reactivation	
	(AchE, human blood, in vitro, variation limits for all intervals studied)	(AChE, dog blood, in vivo, means with their confidence limits, $p \leq 0.05$)
Soman	0 - 10	16,06 _ 6,06
Sarin	15 - 63	62,67 _5,79
VX	55 - 85	87,61 _8,79

DISCUSSION

Our results dealing with reactivatability of the blood AChE following soman, sarin and VX intoxication showed that this approach is suitable as that used for insecticides. Our results in vitro and in vivo are in good correlation. The reactivation test would be useful also for OP in forensic chemistry. If we compare the results in vivo, it can be concluded that the test can be used not only for determination of reactivatability but also for differential diagnosis of intoxications with soman: reactivation about 50 % demonstrates intoxication with sarin and higher reactivation than 80 % might be typical for VX intoxication.

From the results presented, it can be assumed halftime of dealkylation, for soman it lies in the range of 5-10 min, for sarin it is more than 2 hours and for VX dealkylation was not observed within 2 hours. This is in excelent agreement with literature data (2,3,5).

We developed method for double AChE determination in the blood - the first one without and the second one with reactivator. When the activity in the second determination is the same or lower than 0 - 10%, then soman intoxication is very suspect and repeated administration of reactivators will be uneffective. When reactivation is observed in the range of 30 - 50 %, very probably, organism was poisoned with sarin. In case of 80 % and higher reactivation, VX is probably the toxic agent. In these cases, administration of reactivators is indicated.

SUMMARY

Nerve agents inhibit acetylcholinesterase (AChE, EC 3.1.1.7.) in the erythrocytes and cholinergic nervous system. The basic therapeutic interventions comprise administration of atropine (paralyzing effects of accumulated neuromediator acetylcholine) and reactivators of AChE (reactivating by nerve agent inhibited AChE). However, in some cases, depending on the structure of nerve agent, inhibited AChE is changed to complex resistant to reactivators. It is unreactivatable. In these cases, repeated administration of reactivators is not effective. This reaction called aging or dealkylation is very fast for soman ($t_{0,5}$ approximately 10 - 12 hours) and for VX, the $t_{0,5}$ was more than 24 hours.

We developed method for double AChE determination in the blood - the first one without and the second one with reactivator. When the activity in the second determination is the same or lower than 0 - 10 %, then soman intoxication is very suspect and repeated administration of reactivators will be uneffective, when reactivation is observed in the range of 30 - 50 %, very probably, organism was poisoned with sarin. In case of 80 % and higher reactivation, VX is probably the toxic agent. Reactivators are indicated. The test was verified by experiments in vitro (human blood) and in vivo on dogs using sarin, soman and VX.

Support of the NATO - Linkage grant (DISRM.LG 960581) is gratefully acknowledged.

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KEYWORDS

erythrocyte acetylcholinesterase, sarin, soman, VX, in vitro, human, in vivo, dog

23. THE LONG-TERM EFFECTS OF LOW LEVEL INHALATION EXPOSURE OF RATS TO SARIN

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INTRODUCTION

Highly toxic organophosphates (OPs), called as nerve agents, represent potential threats to both military and civilian population, as evidenced in recent terrorist attacks in Japan (1). The irreversible binding to and subsequent inactivation of acetylcholinesterase (AChE, EC 3.1.1.7), the enzyme that normally catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) at neuromuscular junctions and other cholinergic synapses, is generally believed to be the major mechanism of OP poisoning. The accumulation of ACh in the cholinergic clefts causes the overstimulation of peripheral as well as central cholinergic nervous system, clinically manifested as acute cholinergic crisis (2, 3). The exposure to high, convulsive doses of nerve agents can result in severe brain pathology (4).

In addition, OPs have many other effects that have an influence on the functions of various organs and systems of organs. They are called as non-specific or non-cholinergic effects and involve the activation of multiple non-cholinergic neurotransmitter systems in the central nervous system, mutagenic, stressogenic immunotoxic, hepatotoxic, membrane and hematotoxic effects (5).

Nerve agents-induced cholinergic as well as non-cholinergic effects are usually manifested immediately following high-level or intermediate-level exposures to these chemical warfare agents (2, 3). Nevertheless, there are numerous studies in both humans and animals showing that survivors of high-level and possibly intermediate-level exposure acute poisoning by nerve agents can experience subtle but significant long-term neurological and neuropsychological outcomes that are detectable months or even years following the recovery from acute poisoning (6). On the other hand, very little is known about possible subtle neurological and other effects of low-level, asymptomatic exposures to nerve agents. Incidents, such as terrorist attacks against Japanese civilians (1) and the possible low-level exposure to military personnel during the 1991 Gulf War from nerve agents (7), have increased the interest in the research dealing with the evaluation of the long-term influence of low-level exposure to OPs on various physiological functions. The purpose of this study is to find out whether a nerve agent, such as sarin, might cause long-term health effects following the single or repeated low-level inhalation exposure in rats.

MATERIALS AND METHODS

Animals: Male albino Wistar rats weighing 180-220 g were purchased from VÚFB Konárovice (Czech Republic). They were kept in an air-conditioned room and allowed to access to standard food and tap water ad libitum. Food as well as water were sterilized before their use. The rats were divided into groups of ten animals. Handling of the experimental animals was done under the supervision of the Ethics Committee of the Medical Faculty of Charles University and the Military Medical Academy in Hradec Králové (Czech Republic).

Chemicals and drugs: Sarin was obtained from Zemianské Kostolany (Slovak Republic) and was 98.5% pure. All other chemicals of analytical grade were obtained commercially and used without further purification.

Animal experiments: Rats were exposed to low concentrations of sarin for 60 minutes in the inhalation chamber. Three concentrations of sarin were chosen:

- LEVEL 1: clinically and laboratory asymptomatic concentration (0.8 µg/L)
- LEVEL 2: clinically asymptomatic concentration with significant inhibition of erythrocyte AChE by 30% (1.25 µg/L). This concentration was used for a single (LEVEL 2S) or repeated (three times during one week) exposure (LEVEL 2R).
- LEVEL 3: non-convulsive symptomatic concentration (2.5 µg/L)

At three months following the exposure to sarin, the rats were killed by exsanguination in total anesthesia to evaluate chosen parameters of sarin-induced health effects. The hematological parameters were evaluated in the blood using an automated hematology analyzer (8). The biochemical parameters were measured in serum using various commercial kits. The biochemical evaluation included the measurement of cholinesterase activities in erythrocytes, plasma, diaphragm and brain by the spectrophotometric method (9).

Two methods were used to evaluate the possible sarin-induced alteration of immune functions:

- in vitro spontaneous or lipopolysaccharides (LPs)-stimulated proliferation of spleen cells (lymphocytes) that was evaluated by measurement of the synthesis of nucleic acid with the help of incorporated [^{3}H] thymidine (10).
- in vitro evaluation of the production of reactive nitrogen intermediates (N-oxides) that reflects the bactericidal efficacy of peritoneal macrophages (11).

To evaluate sarin-induced signs of neurotoxicity at three months following the inhalation exposure, other groups of control and experimental rats were monitored using a functional observatory battery (FOB), a non-invasive and relatively sensitive type of neurobehavioral examination including measurements of sensory, motor and autonomic nervous functions (12, 13) and a test of excitability of central nervous system by the observation of a convulsive activity following i.p. administration of pentamethylenetetrazol (PTZ) at a convulsive dose (30 mg/kg). Occurrence of abnormal signs, especially the features and intensity of convulsions was evaluated according to a specially elaborated scale (14).

Data analysis: Statistical analyses were performed on a PC with BMDP program P7D: analysis of variance (ANOVA) and t-test with Bonfferi`s corrections. The differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Sarin–exposed rats did not show any clinical signs of intoxication and their body weight did not differ significantly from control values at three months following the inhalation exposure. The values of their hematological parameters in blood and biochemical parameters in serum are summarized in tables 1 - 2. We found that all measured hematological and biochemical parameters from sarin-exposed rats did not differ from control values regardless of the concentration of sarin used (TAB 1, 2). Similarly, no changes in the activities of cholinesterase in erythrocytes, plasma, diaphragm and brain in comparison with control values were observed (TAB 3). Thus, not only asymptomatic but also non-convulsive symptomatic dose of sarin is not able to cause significant laboratory changes in homeostasis of sarin-exposed rats lasting at least three months following the inhalation exposure. It corresponds to earlier demonstrated findings that severely poisoned patients during the terrorist attacks with sarin in Matsumoto also suffered from changes in biochemical and hematological parameters for a relatively short time. The AChE level of these sarin-poisoned patients returned to normal within three months (15).

The results of the evaluation of sarin-induced alteration of immune functions at three months following low-level sarin inhalation exposure are shown in figure 1. The significant decrease ($p < 0.05$) in LPs-stimulated lymphoproliferation as well as the production of N-oxides (macrophage activity) in comparison with the control values was observed when the experimental rats were exposed to non-convulsive concentration of sarin (LEVEL 3). The spontaneous lymphoproliferation was also decreased following the exposure to sarin at LEVEL 3 but this decrease was not significant. On the other hand, asymptomatic concentrations of sarin were not able to cause significant alteration of immune functions studied at three months following the exposure in comparison with the control values (FIG 1). Thus, on contrary of hematological and biochemical parameters, sarin-induced alteration of some immune functions can last for a relatively long time although the level of sarin exposure was not too high (clinically symptomatic, but non-convulsive). The immunotoxic effects of OP compounds were already demonstrated earlier, but usually followed the intoxication with high doses of OP insecticides (16).

The results of the measurement of sarin-induced signs of neurotoxicity at three months following low-level sarin inhalation exposure of rats are summarized in table 4 and figure 2. The alteration of mobile activity and gait characterized by ataxia and the increase in stereotyped behavior were observed in rats exposed to sarin at asymptomatic or non-convulsive symptomatic level ($p < 0.05$) (TAB 4). Moreover, the significant increase in the excitability of central nervous system characterized by the higher incidence of small and big seizures and other convulsive performances following the administration of PTZ was monitored in rats repeatedly exposed to sarin at LEVEL 2 ($p < 0.05$) (FIG 2). Thus, long-term sarin-induced neurobehavioral and neurophysiological effects were demonstrated in rats exposed to low-level sarin without a significant inhibition of AChE activity and a clinically manifested alteration of cholinergic nervous system. The findings correspond with earlier published data about neurological and neurophysiological outcomes detectable months or even years following recovery from acute poisoning (17,18). It means that probably other than cholinergic nervous system can be involved in nerve agent-induced long-term signs of neurotoxicity. It has been reported that there are protein targets present in brain, which are known to be very sensitive to some anticholinesterase compounds including the nerve agents. They may represent a target for OP-induced low-level effects, either adverse or beneficial. However, the functions of these protein targets have not been yet known (19).

In conclusion, although these findings are difficult to extrapolate directly to human low-level exposures to nerve agents, they indicate that subtle neurophysiological and behavioral dysfunction and alteration of immune functions without disturbance of cholinergic nervous system could also occur in humans months following the inhalation exposure to non-convulsive symptomatic or even repeated inhalation exposure to asymptomatic level of sarin.

SUMMARY

Male albino Wistar rats were exposed to low concentrations of sarin for 60 minutes in the inhalation chamber. Three concentrations of sarin were chosen:

• LEVEL 1: clinically and laboratory asymptomatic concentration (0.8 μg(L)

• LEVEL 2: clinically asymptomatic concentration with significant inhibition of erythrocyte acetylcholinesterase by 30% (1.25 μg/L)

• LEVEL 3: non-convulsive symptomatic concentration (2.5 μg/L)

In addition, one group of experimental animals was exposed to clinically asymptomatic concentration of sarin repeatedly (three times during one week). The clinical status of poisoned rats was tested 3 months following exposure to sarin using biochemical, hematological, neurophysiological, behavioral and immunotoxicological methods.

While biochemical and hematological parameters, including the activities of cholinesterases in erythrocytes, plasma and various organs (brain, diaphragm), did not differ from the control values regardless of the sarin concentration used, some signs of sarin-induced neurotoxicity and immunotoxicity in sarin-poisoned rats were demonstrated. This was especially true when the exposure of rats to non-convulsive symptomatic concentrations of sarin and clinically asymptomatic concentrations of sarin was repeatedly used. In rats repeatedly poisoned at clinically asymptomatic concentrations of sarin, the alteration of the gait characterized by ataxia, the increase in the stereotyped behavior, the increase in the excitability of the central nervous system following the administration of the convulsive drug pentamethylenetetrazol were observed. In rats poisoned with non-convulsive symptomatic concentrations of sarin, the suppression of spontaneous, as well as LPs-stimulated, proliferation of spleen lymphocytes and the bactericidal activity of peritoneal macrophages was primarily observed besides the signs of neurotoxicity.

Our findings confirm that both non-convulsive symptomatic and clinically asymptomatic concentrations of sarin can cause long-term signs of neurotoxicity and immunotoxicity in sarin-poisoned rats when the rats were exposed to asymptomatic sarin concentrations repeatedly.

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KEYWORDS
Sarin, inhalation exposure, rat, neurotoxicity, immunotoxicity

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FIGURES AND TABLES

Figure 1. The alteration of immune functions in sarin-poisoned rats at three months following the inhalation exposure.

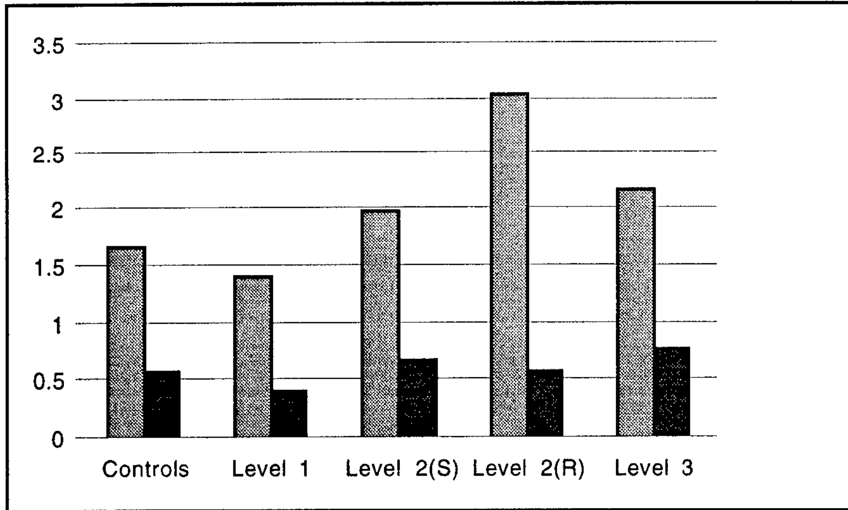

Figure 2. The excitability of CNS in sarin-poisoned rats at three months following the inhalation exposure.

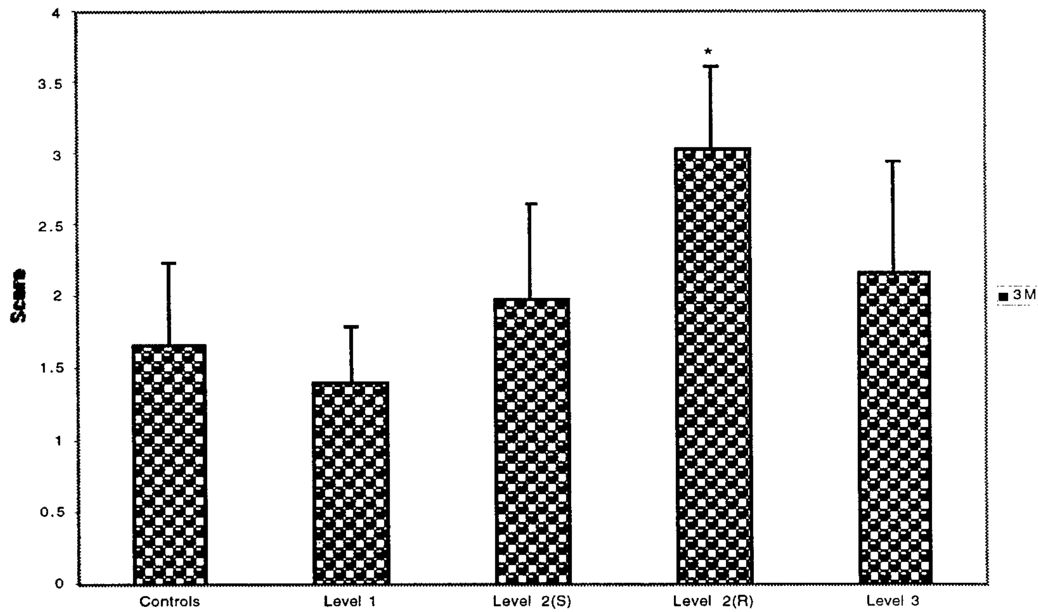

Table 1. Hematological parameters in sarin–poisoned rats at three months following the inhalation exposure.

Level of sarin exposure	Control	Level 1	Level 2(S)	Level 2(R)	Level 3
Number of erythrocytes (1012/L)	7.89 ± 0.2	8.04 ± 0.2	7.97 ± 0.2	8.08 ± 0.2	8.00 ± 0.2
Hemoglobin (g/L)	151.1 ± 4.0	151.1 ± 4.0	151.2 ± 4.0	156.6 ± 4.0	154.9 ± 4.0
Hematocrit	0.429 ± 0.105	0.423 ± 0.105	0.427 ± 0.105	0.429 ± 0.105	0.423 ± 0.105
Number of leukocytes (109/L)	4.0 ± 0.8	5.1 ± 0.8	5.5 ± 0.8	4.4 ± 0.8	4.2 ± 0.8
Number of lymphocytes (%)	94.5 ± 1.1	94.4 ± 1.1	94.2 ± 1.1	95.3 ± 1.1	93.3 ± 1.1
Number of monocytes (%)	4.3 ± 0.8	4.4 ± 0.8	4.5 ± 0.8	3.7 ± 0.8	5.1 ± 0.8
Number of granulocytes (%)	1.14 ± 0.36	1.25 ± 0.36	1.35 ± 0.36	1.05 ± 0.36	1.61 ± 0.36
Number of thrombocytes (109/L)	756 ± 51	733 ± 51	775 ± 51	813 ± 51	770 ± 51

Table 2. Biochemical parameters in sarin-poisoned rats at three months following the inhalation exposure.

Level of sarin exposure	Control	Level 1	Level 2(S)	Level 2(R)	Level 3
Total protein (g/L)	65,0 ± 1,8	65,4 ± 1,8	65,9 ± 2,2	65,2 ± 2,4	64,8 ± 1,4
Albumin (g/L)	37,5 ± 0,8	37,5 ± 0,8	37,6 ± 0,8	37,1 ± 1,2	38,4 ± 0,6
Glycemia (mmol/L)	8,60 ± 1,2	8,74 ± 0,6	9,64 ± 0,5	8,98 ± 0,9	9,39 ± 0,9
Cholesterol (mmol/L)	1,44 ± 0,19	1,42 ± 0,22	1,45 ± 0,15	1,71 ± 0,22	1,54 ± 0,20
Triacylglycerol (mmol/L)	1,60 ± 0,50	1,80 ± 0,76	1,70 ± 0,50	1,21 ± 0,47	1,12 ± 0,61
Total bilirubin (mmol/L)	1,61 ± 0,24	1,53 ± 0,22	1,60 ± 0,23	1,46 ± 0,21	1,42 ± 0,28
AST (mkat/L)	1,86 ± 0,25	1,64 ± 0,20	1,76 ± 0,19	1,64 ± 0,20	1,67 ± 0,20
ALT (mkat/L)	0,82 ± 0,20	0,68 ± 0,05	0,83 ± 0,15	0,64 ± 0,08	0,79 ± 0,16
GGTP (mkat/L)	0,19 ± 0.01	0,20 ± 0,01	0,19 ± 0,01	0,20 ± 0,01	0,19 ± 0,01
ALP (ukat/L)	2,01 ± 0,46	1,74 ± 0,23	1,96 ± 0,29	1,98 ± 0,32	1,82 ± 0,24
Na+ (mmol/L)	145,3 ± 2,1	145,9 ± 1,4	143,5 ± 1,4	147,4 ± 2,8	143,0 ± 1,9
K+ (mmol/L)	4,06 ± 0,62	3,99 ± 0,18	3,97 ± 0,15	3,88 ± 0,34	4,13 ± 0,48
Cl- (mmol/L)	100,9 ± 1,4	102,2 ± 1,0	100,4 ± 0,9	102,8 ± 2,1	99,8 ± 1,0
Urinary acid (mmol/L)	33,9 ± 13,2	25,9 ± 7,6	42,9 ± 17,4	43,5 ± 12,0	45,3 ± 18,8
Urea (mmol/L)	8,13 ± 0,96	7,55 ± 1,10	8,14 ± 0.99	9,19 ± 1,32	9,17 ± 1,14
Creatinin (mmol/L)	54,3 ± 2,5	54,5 ± 3,6	55,9 ± 3,6	57,2 ± 3.5	78,7 ± 3.8

Table 3. Activities of cholinesterases in sarin-poisoned rats at three months following the inhalation exposure.

Level of sarin exposure	Control	Level 1	Level 2(S)	Level 2(R)	Level 3
AChE in erythrocytes	100 ± 9,8	97,4 ± 9,8	98,9 ± 9,8	103,7 ± 9,8	103,6 ± 9.8
BuChE in plasma	100 ± 12,2	109,6 ± 12,2	110,8 ± 12,2	95,0 ± 12,2	93.1 ± 12.2
AChE in diaphragm	100 ± 8,0	112,9 ± 8,0	105,5 ± 8,0	114,0 ± 8,0	108.4 ± 8.0
AChE in brain	100 ± 10,2	101,0 ± 7,1	103,4 ± 7,1	100,4 ± 7,1	100,8 ± 7,1

Table 4. The values of sarin-induced neurotoxic markers (x ± s) measured by FOB (1-36 scored values, 37-45 – values in absolute units) at three months following the inhalation exposure. Statistical significance - * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

		Controls		Level 1		Level 2 (S)		Level 2 (R)		Level 3	
		x	±s	x	±s	x	±s	x	±s	x	±s
1	posture	2	0	2	0	3.2	0.32	2	0	2	0
2	catch difficulty	2.1	0.31	1.4	0.26	2	0.33	1.3	0.21	1.2	0.2
3	ease of handling	1.2	0.2	1.4	0.26	1	0	1.2	0.2	1.2	0.2
4	muscular tonus	1	0	1	0	1	0	1	0	1	0
5	lacrimation	1	0	1	0	1	0	1	0	1	0
6	palpebral closure	1	0	1	0	1	0	1	0	1	0
7	endo-exophthalmus	1	0	1	0	1	0	1	0	1	0
8	piloerection	1	0	1	0	1	0	1	0	1	0
9	skin abnormalities	1	0	1	0	1	0	1	0	1	0
10	salivation	1	0	1	0	1	0	1	0	1	0
11	secretion	1	0	1	0	1	0	1	0	1	0
12	exploratory activity	7.4	1.22	7.2	1.24	4	1.35	8.5	1.77	9.2	1.45
13	urination	1.7	0.87	2.7	1.64	1.2	0.99	5.3	1.68	4.7	1.18
14	defecation	0.2	0.2	1.4	0.56	0.5	0.4	1.3	0.53	0.8	0.35
15	clonic convulsions	1	0	1	0	1	0	1	0	1	0
16	tremor	1	0	1	0	1	0	1	0	1	0
17	tonic convulsions	1	0	1	0	1	0	1	0	1	0
18	gait disorder	1	0	1	0	1	0	2,00***	0.33	2,00***	0.33
19	gait score	1	0	1.5	0.26	30	0.47	0.8	0.32	-2,00**	0.58
20	mobility score	1	0	1.5	0.26	30	0.47	-2,00**	0.58	-2,00**	0.58
21	activity	1	0	1.5	0.26	30	0.47	1.2	0.44	-2,00**	0.58
22	tension	1	0	1	0	1	0	1	0	1	0
23	vocalizations	2.8	0.2	3	0	3	0	2.8	0.2	2.4	0.4
24	stereotypy	1	0	1.5	0.5	2,00*	0.66	6,00***	0	4,00**	0.8
25	bizarre behavior	1	0	1	0	1	0	1	0	1	0
26	approach response	1	0	1.1	0.1	120	0.16	1	0	1	0
27	touch response	1.6	0.16	1.9	0.1	-1.7	0.15	1.9	0.1	1.9	0.1
28	click response	1.3	0.3	1	0	-2.1	0.37	1.1	0.1	1	0
29	tail-pinch response	2.6	0.3	1	0	-2.2	0.41	1.1	0.1	1.1	0.1
30	pupil size	1	0	1	0	1	0	1	0	1	0
31	pupil response	1	0	1	0	1.2	0.2	1	0	1	0
32	righting reflex	1	0	1	0	1	0	1	0	1	0
33	fall from vertical position	1	0	1	0	1	0	1	0	1	0
34	landing foot splay (cm)	90.8	4.88	109.6	9.37	98.8	4.73	109	5.98	92.4	3.7
35	forelimb grip strength (kg)	12.92	0.99	11.69	0.96	12.69	0.94	11.61	0.92	12.4	0.59
36	hindlimb grip strength (kg)	6.28	0.46	5.98	0.22	6.52	0.48	6.66	0.68	5.69	0.35
37	fore and hindlimb grip strength (kg)	29.03	1.83	31.19	1.58	26.51	2.22	28.87	1.39	29.12	1.15
38	body weight (g)	347.3	7.46	349.2	9.52	346.1	5.13	350.3	9.31	347.9	8.92
39	rectal temperature (°C)	38.34	0.06	37.82	0.12	38.19	0.05	38.24	0.12	38.5	0.11
40	damage of respiration	1	0	1	0	1	0	1	0	1	0
41	activity horizontal (No/10 min)	355.2	49.21	375.2	36.68	336.8	30.61	398.4	20.38	375.6	35.88
42	activity vertical (No/10 min)	170.8	35.58	116.4	25.73	141.9	27.01	153	18.18	136.8	20.77

24. PLAGUE VACCINES: AN OVERVIEW

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ABSTRACT

Despite being a comparatively rare disease, endemic foci of plague are widely distributed throughout the world. Plague has been a concern for military forces in some theatres of operations in the past and remains a potential problem for future deployments. These concerns are further compounded by fears that plague may be deliberately released as a biological warfare weapon.

Protection of armed forces personnel can be achieved through vaccination. There are essentially three current plague vaccines, a heat-killed preparation, a chemical-killed vaccine and a live attenuated vaccine. The origins of these vaccines can be found from the turn of the century following the discovery of *Yersinia pestis*, the bacteria causing plague and their protective efficacy is based upon an antibody response to the fraction 1 (F1) antigen, a key virulence determinant of *Y. pestis*.

There are a number of problems associated with these vaccines in terms of limited availability, production, and side effects. Protective efficacy has never been proven in randomised, controlled clinical studies although animal studies and epidemiological studies of vaccinated personnel in Vietnam between 1961-1971 suggest the vaccines are protective against the bubonic form of the disease. The protection offered by the killed vaccines against pneumonic plague is questionable. The live vaccine does protect against pneumonic plague but the vaccine is associated with severe side effects. In either case the protection is short lived and frequent boosters are required.

Newer sub-unit vaccines based on F1 antigen and a second virulence determinant, the V antigen, and live recombinant vaccines show promise in providing long-lasting protection against both bubonic and pneumonic plague with fewer side effects.

INTRODUCTION

Despite being a comparatively rare disease, plague is associated with past pandemics which caused unparalleled death and social upheaval wherever it occurred. The causative organism *Yersinia pestis* was carried throughout the world by the rodent reservoir in three pandemics. The first pandemic spread throughout the Mediterranean during the 6-8th centuries, the second occurred throughout the Eurasian land mass during the 14th century and during the third pandemic the disease finally reached the New World at the end of the 19th century. The fear of plague remains exemplified by the national and international response to a plague outbreak in India in 1994. The final pandemic has resulted in broad enzootic foci throughout the world[1].

Military operations in the past have encroached on these areas, for example, the Far East campaign during the Second World War, and the Vietnam conflict during the 1960s. Current military operations, particularly peace-keeping operations and humanitarian relief operations, not only place military personnel within plague foci but also among populations living in conditions conducive to an outbreak of plague. Furthermore, there is possible exposure to the rodent reservoir in some of the large training areas and there is a fear of plague being deliberately used a biological weapon.

Plague generally is a disease of rodents and rodent-like mammals. In the urban environment both the black rat and brown rat are the principal reservoirs. In sylvatic plague, numerous species can act as reservoirs including squirrels, chipmunks, marmots and prairie dogs. The disease is passed between individual animals by a flea vector, although *Y. pestis* is particularly adapted to carriage by certain species of flea, the most notable being the Oriental rat flea, *Xenopsylla cheopsis*. The flea takes a blood meal from an infected animal. *Y. pestis* causes the blood to coagulate and block the feeding parts of the flea forcing the flea to attempt to feed more frequently. With each bite a piece of the plague-laden clot is deposited in then wound. Humans are occasionally bitten by the fleas when there is close contact between the animal reservoir and humans either through poor living conditions or through occupational exposure[2].

Following a bite, *Y. pestis* spreads from the wound to the lymphatic system then to a local lymph node causing painful swelling - the "buboe" of bubonic plague. Occasionally, the organism spreads to the lungs via the circulatory system giving rise to a secondary pneumonic disease which can be transmitted

from person-to-person by aerosols generated by coughing and sneezing. The disease may also result from direct contact or ingestion of contaminated animal material[2].

VACCINE HISTORY

Very quickly following the discovery of *Y. pestis* in 1894 by Alexandre Yersin, efforts were made in producing a vaccine. By 1897, Haffkine had prepared a crude killed preparation which became the basis of the current killed vaccine. At the time, however, Haffkine's original vaccine was associated with severe side effects (considered to be indicative of protection) with some fatalities and eventually withdrawn[3].

Studies in the USA preceding the Second World War found that reactogenicity of killed vaccine was associated with the bacterial load contained in the vaccine. By 1941 a formaldehyde-killed formulation was available which became the Plague Vaccine, USP[3]. Initially, Plague Vaccine, USP was manufactured by Cutter Incorporated until 1995 when Greer Laboratories undertook production. Essentially the Cutter/Greer formulations were identical.

The efficacy of attenuated strains of *Y. pestis* as live vaccines was considered almost immediately following the identification of *Y. pestis.* Yersin and others carried out animal studies but found that most attenuated strains were capable of reverting and causing plague in their animal models. In 1908 Strong immunised humans with strain "Maassen", and during the 1930s, Girard and Robic used strain EV in Madagascar, and Otten used strain Tjiwidej in Java[4]. A live vaccine consisting of strain EV76 was used widely up until 1970.

CURRENT VACCINES

All of the current plague vaccines are associated with problems regarding production, availability, licensing and side effects. Production requires specialised containment facilities making vaccine manufacture expensive. Low demand for the vaccine undermines commercial viability which in turn determines availability. Each of the vaccines is a crude preparation and all cause side effects varying in severity. Pharmaceutical legislation, therefore, ultimately dictates which vaccines are available to a particular nation. In the UK two killed preparations were available until 1999; the formaldehyde-killed Plague Vaccine, USP produced by Greer Laboratories and a heat-killed formulation manufactured by Commonwealth Serum Laboratories (CSL) in Australia. The Plague Vaccine, USP has now been withdrawn and is no longer available and the CSL vaccine is available on a "named-patient" basis.

A formaldehyde-killed preparation may still be produced by the Haffkine Institute in Bombay[5], although the availability is unclear. The live vaccine strain EV76 is no longer used in the west but may still be available in some countries.

PROTECTIVE EFFICACY

The protective efficacy of the killed plague vaccines is based on stimulating an antibody response to a key virulence factor, the Fraction 1 (F1) antigen. Evidence from animal studies suggests that protection correlates to the anti-F1 titre[6]. Protection is short-lived, necessitating frequent booster immunisations (6 monthly-annually), and there is poor protection against pneumonic plague (Figures 1 and 3).

Strain EV76 is a pigmentation-deficient variant of *Y. pestis*, capable of expressing a number of virulence factors in addition to F1 antigen, which may serve as antigens, including V antigen (known to be protective), secreted proteins and lipopolysaccharide (LPS)[7]. The production of these virulence factors, absent in the killed preparations, contributes to better protection, including some protection against pneumonic plague, although like the killed vaccines, protection is short-lived.

Despite the apparent benefits of the live vaccine, there is wide variation in the immunogenicity and virulence between different EV76 vaccine preparations. Severe side effects in humans and the inability to precisely define the genetic basis of the attenuation has largely precluded its use as a vaccine.

None of the vaccines have been subjected to randomised clinical trials in humans. The evidence for protection is largely derived from animal trials (Figure 1) and limited epidemiological studies. The earlier vaccination programmes instituted by Haffkine, Otten, Girard and Robic did reduce incidence during outbreaks. Between 1961-1971 US service personnel serving in Vietnam were immunised with a combination of the live and killed vaccine. Incidence in service personnel was reduced to a single case per 10^6 person-years exposure compared to 333 cases per 10^6 person-years exposure among the local

population[8]. A mass immunisation programme was initiated for the population but the effectiveness of the programme was never determined.

FUTURE VACCINES

The current vaccines are based on formulations derived from the 1930s when plague was a far greater problem than it is now. The lack of commercial return has largely precluded any development of the vaccine in the civilian sector and research has generally been undertaken by military agencies. The threat of plague remains furthermore, the appearance of antibiotic resistant plague has highlighted the need for a vaccine to counter future outbreaks.

The current vaccines indicate that immunoprophylaxis against plague is feasible but modern biotechnological and recombinant techniques can facilitate production, reducing costs, and provide a purer formulation which should have less side effects. F1 antigen is clearly an important component of a vaccine[9] and recombinant F1 antigen produced in *Escherichia coli* is capable of protecting mice to the same degree as the native protein. V antigen has been recognised as another important protective antigen although the protein is produced at low levels by *Y. pestis* and is difficult to purify. Recombinant technology has enabled sufficient quantities of V antigen to be evaluated as a vaccine. V antigen provides a higher degree of protection compared to F1 antigen, but when both antigens are combined, they act synergistically to provide very high levels of protection against systemic and pneumonic plague in mice which is long lasting (Figures 2 and 3). Recombinant technology also permits a way forward for live vaccines using defined attenuated gram negative vectors expressing *Y. pestis* antigens.

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KEYWORDS
Plague, *Yersinia pestis*, vaccine, efficacy

FIGURES AND TABLES

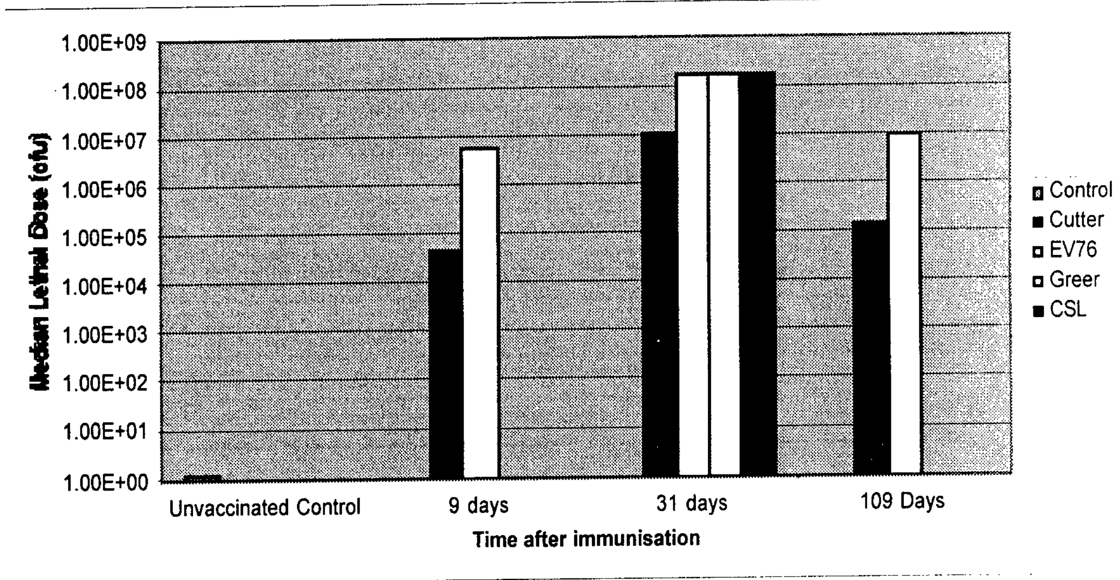

Figure 1: Comparison of efficacy of plague vaccines in a murine model. In each case immunisation consisted of two doses of approximately 10^8 cells given 7 days apart by intraperitoneal injection. Mice were challenged with *Yersinia pestis* strain GB by subcutaneous injection.

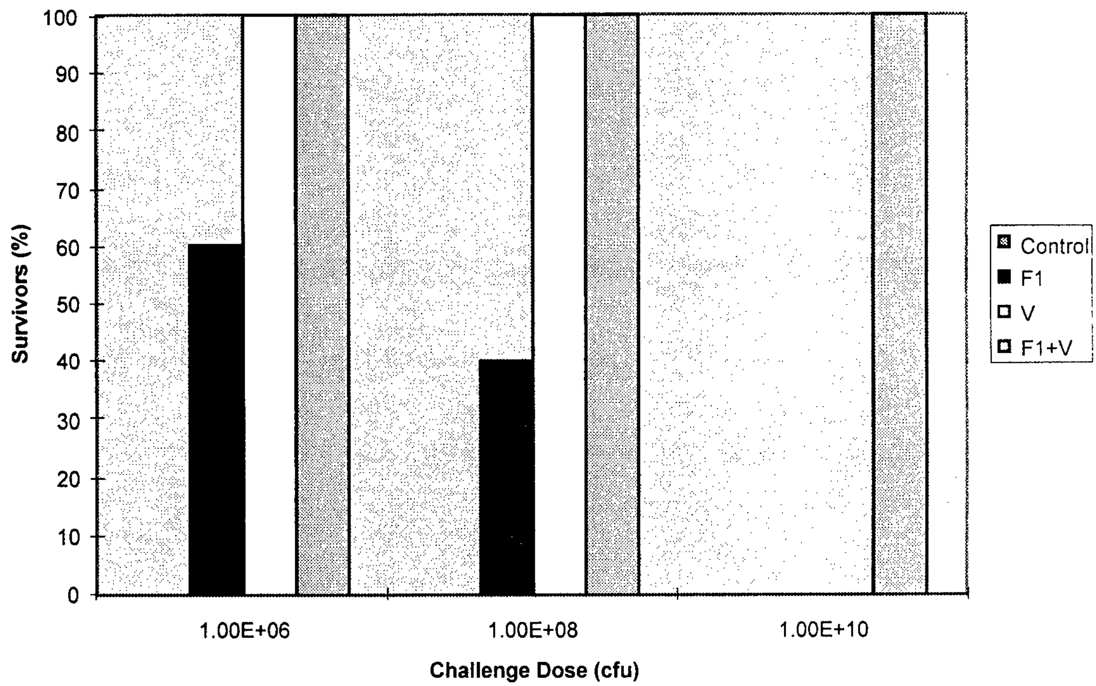

Figure 2: Additive protective efficacy of F1 and V antigens formulated as a vaccine against systemic *Yersinia pestis* in mice[10].

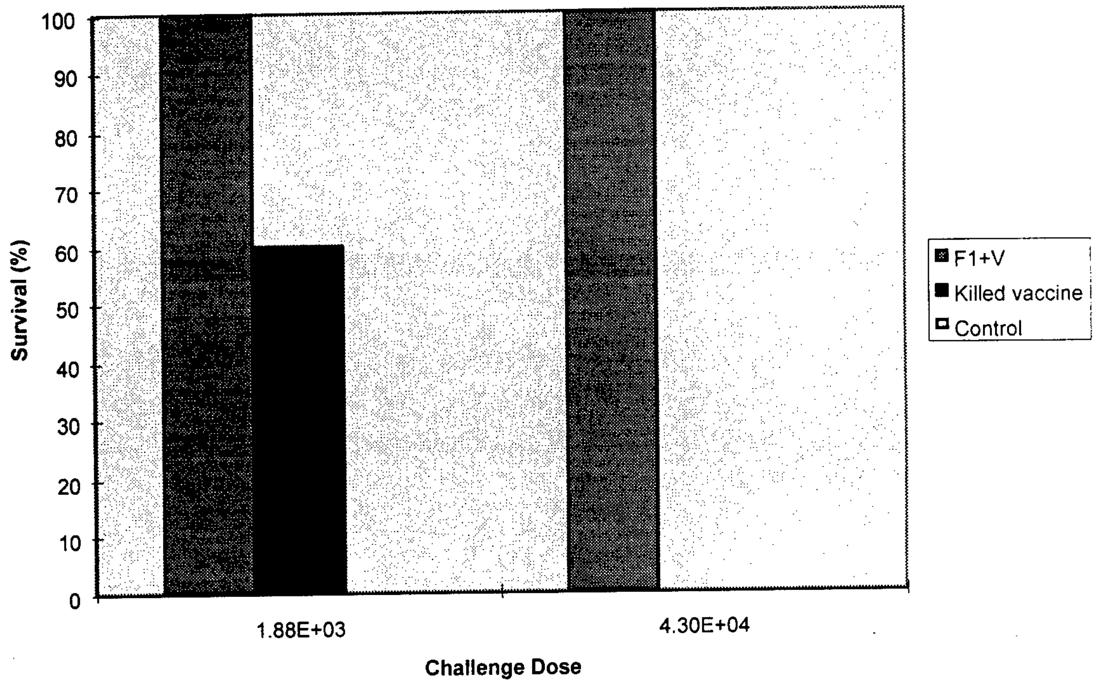

Figure 3: Efficacy of sub-unit vaccine based on F1 and V antigen compared to a killed vaccine against an experimental pneumonic infection of *Yersinia pestis* in mice[11].

25. ASSESSING THE EFFECTS OF LOW DOSE EXPOSURE TO ANTICHOLINESTERASES

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ABSTRACT

The effects of exposure to low doses of cholinesterase inhibitors are still poorly understood. Reliance upon human epidemiological evidence to assess the effects of such exposure is unsatisfactory because of the uncertainty associated with exposure levels and the potential complication of a plethora of pre-disposing factors. There is, therefore, a requirement to conduct focused research in relevant animal models which optimise the confidence with which animal-derived data can be extrapolated to man. Such an approach will provide a scientifically sound context for the interpretation of epidemiological investigations.

Burchfiel et al (1976) suggested that, in non-human primates, a sign-free dose of sarin, induced small but statistically significant changes in aspects of the electroencephalogram (EEG) although the functional significance of these changes was not known. Recent studies at CBD have led to the development of a sophisticated, multifaceted non-human primate model, which revisits and extends these earlier studies.

The model has already been used to investigate the effects of a similar dose of sarin on EEG, sleep and cognitive function in marmosets. Pearce et al, (1999) did not replicate the previously reported EEG changes and found no decrement in cognitive performance over the 15-month study duration The model offers considerable potential for studying the effects of a wider dose range of sarin and other compounds of interest.

The approach adopted and the results of the study will be described in detail as well as related work in progress and opportunities for the future.

BACKGROUND

There is widespread concern in both military and agricultural communities as to whether acute or chronic exposure to low doses of organophosphate (OP) anticholinesterase compounds, at doses which do not produce marked clinical signs, give rise to adverse long term effects. While the acute toxicity of OP compounds is well characterised (e.g. McLeod. 1985) long term effects relating to central functioning are poorly understood. Many studies have suggested that administration of OPs produce effects in the central nervous system that persist for many weeks or months (e.g. Grob & Harvey. 1953; Steenland, 1996). Typical symptoms which have been reported include tension, anxiety, difficulty in concentrating, slowness of recall, mental confusion and sleep disturbances (e.g. Sherman, 1995). The majority of these studies, however, have lacked precise information on the level of OP exposure, the degree of acetylcholinesterase (AChE) inhibition and the time course of sequelae. For example, Bowers et al (1964) investigated 93 Army or Air Force personnel exposed to OP and found changes in a variety of psychological parameters. However, no quantitative testing of cognitive function was carried out. Follow up of victims of the sarin attack on the Tokyo underground in 1995 has suggested long term effects on behaviour may have occurred in some people (Yokoyama et al 1998).

The rapid onset of signs and symptoms of poisoning following OP exposure can be explained in terms of acetylcholine accumulation following cholinesterase inhibition but no mechanism has been identified for the induction of long term effects. Experimental studies in non-human primates have suggested that administration of clinically sign free doses of the OP sarin (isopropyl methylphosponofluoridate) can give rise to subtle but statistically significant changes in brain electrical activity, as gauged by electroencephalography (EEG). Burchfiel, Duffy & Sim (1976) demonstrated a significant increase in the fast beta component (13-50 Hz) of the EEG spectrum in response to both a large acute and repeated small doses of sarin in rhesus monkeys. In this study however, EEG was monitored only at 24 h and 12 months following dosing and cholinesterase assessments were not reported. The functional significance of these changes is not known.

The object of the study recently conducted at CBD (Pearce et al, 1999) was to ascertain whether administration of an acute low dose of sarin would give rise to long-term EEG changes in common marmosets (Callithrix jacchus). In order to assess the functional significance of any EEG changes which might be observed, the animals were trained to perform complex behavioural test sequences. Regular monitoring of AChE activity was also incorporated into the study design which was based upon, and represents a refinement of, earlier unpublished preliminary studies conducted in a small number of rhesus monkeys at CBD. Marmosets were selected for the present study because of their increasingly widespread use in neuropsychological investigations (e.g. Roberts et al, 1988; Crofts et al, 1995; Pearce et al. 1998; Ridley et al, 1996). Their small size and species characteristics make them particularly suitable for long-term studies.

The behavioural test employed was modified from human and animal studies and is based upon one element of the Cambridge Neuropsychological Test Automated Battery (CANTAB). It involves the presentation of a number of visual discriminations on a touch sensitive screen and is derived from the Wisconsin Card Sort Test (Grant & Berg, 1948). A number of rule changes are incorporated which are differentially sensitive to a range brain lesions (e.g. Roberts et al, 1992) and pharmacological interventions (Sahakian & Coull, 1993). Tests from this battery are suitable for presentation to both humans and non-human primates and the sequence of stages was adapted from previous human (e.g. Sahakian et al, 1990) and primate (Roberts et al, 1988) studies to facilitate long term repeated presentation. A home cage approach to testing was employed, which has previously been shown to be practicable (Crofts et al, 1995, 1996) and conducive to rapid training and task acquisition (Muggleton et al, 1997).

Traditionally, EEG has been monitored in primates by methods which include the use of restraint chairs (Adams & Barrett, 1974) or backpack and umbilicals (Pearce et al, 1989). In the recent study, EEG measurement was carried out remotely by making use of implantable radiotelemetry which enables acquisition of high quality data over long periods of time with minimal disruption to the animal. The feasibility of this approach has previously been reported elsewhere (Pearce et al, 1996, 1998) and offers considerable advantages over 'traditional' methods of EEG recording, especially in the context of long term studies.

METHODS AND MATERIALS

Animals and diet: Seventeen common marmosets (8 male and 9 female, bred at CBD Porton Down) weighing 319-516g at the beginning of the study were used. All animals were pair housed either in single sex pairs or in mixed sex pairs in which the males were vasectomised. Their daily diet, given after testing, consisted of 20g pellets (Complete primate diet E, Special Dietary Services, Witham, Essex, UK) with supplements of orange segments. On non-testing days, alternative supplements included banana, apple and egg. A sawdust filled tray, in which a small amount of preferred foods (e.g. Rice Krispies, sunflower seeds) was dispersed, allowed the animals to freely engage in foraging behaviour. No form of food deprivation was employed during the study and water was available ad-libitum.

Housing: Housing for each pair consisted of 4 stainless steel cage units measuring H72 x W47 x D60 cm connected together by 2 horizontal external extensions and 1 vertical extension (H18 x W71 x D23 cm and H105 x W 17 x D 23 cm respectively) in order to allow full use of all 4 units. Various items of cage furniture, including hanging wooden dowels, buckets and other playthings, were also placed in the cages. During behavioural training and testing the pairs were separated so that each had use of a single upper unit of its home cage with a rigid extension unit (H18 x W17 x D30 cm) attached to the front. Illumination was provided by sodium lighting, at a level of 350-400 lux at 1 m above the ground, and maintained on a 12 hour light/dark cycle with dusk and dawn effects over 1 hour periods. Temperature was maintained at 25 ^{0}C with 40% humidity.

Drugs: Sarin (isopropyl methylphosphonofluoridate) was synthesised at CBD Porton Down to 95% purity and was stored as 5.0 mg.mL^{-1} solutions in isopropanol. Solutions were diluted to appropriate concentrations with 0.9% saline to give an injection volume of 0.5 mL. 0.9% saline was used for control injects, again in a volume of 0.5 mL.

Overview of experimentd design: The experimental design was centred upon successive presentation of behavioural test sequences over months. The sequence of procedures was conducted in all subjects although the precise time course of the study was dictated by the behavioural performance of individual animals, which served as their own controls.

The first behavioural test sequence was presented after stable baselines of performance had been established. This was followed by surgical implantation of a radiotelemetry transmitter and electrodes which enabled EEG to be recorded at intervals throughout the study. After recovery from surgery, the second and third behavioural test sequences were presented. Control blood samples were taken at intervals to determine baseline levels of erythrocyte cholinesterase activity before administration of sarin or control vehicle. Subsequent to dosing, behavioural test sequences were presented as appropriate.

Administration of either saline or 2.5 or 3.0 μg.kg^{-1} sarin by i.m. injection occurred following completion of the third behavioural sequence. The sarin dose was adjusted to 3.0 μg.kg^{-1} following cholinesterase estimations from the first two subjects dosed which received a dose of 2.5 μg.kg^{-1}. Data from these subjects were included in the analysis as they did not fall outside the range of inhibitions seen for subjects receiving the higher dose. Subsequent to dosing, further behavioural test sequences were presented.

EEG telemetry:The apparatus and surgical procedures used have been fully described elsewhere (Pearce et al, 1998). EEG was collected at least weekly during periods when subjects were undergoing behavioural testing (see below).

BEHAVIOURAL TRAINING AND TESTING

The equipment, training and behavioural test sequences have been described in detail elsewhere (Pearce et al. 1998, Muggleton et al, 1997). After preliminary training, animals were presented with simple discriminations involving pairs of stimuli which consisted of either blue filled 'shapes' or white 'lines' followed by successive presentations of more complex discriminations and reversals. Testing sessions occurred daily Monday-Friday and the maximum session duration was 30 minutes with a maximum of 60 trials per session. At the end of the training phase, subjects were assigned as either 'shape' or 'line' responders. Subsequent test sequences each consisted of a nine stage series of discriminations based on that used by Roberts et al (1988). A criterion of eight successive correct responses determined progression between stages.

Each sequence began with a simple discrimination stage (SD) followed by a simple reversal (SR). Shapes and lines were then combined to form the first compound discrimination (CD) and animals were rewarded for attending to the dimension, shape or line, to which they had initially been assigned. Following presentation of a reversal of this compound discrimination (CR), a pair of novel compound stimuli were presented and animals were rewarded for attending to the same dimension as previously (IDS, intra dimensional shift). Following a reversal (IDR) another pair of novel compound stimuli were presented. At this stage the animals were rewarded for attending to the previously unrewarded dimension (EDS, extra dimensional shift). The sequence finished with a reversal (EDR). This sequence was repeated for the duration of the study using novel stimuli.

Animals underwent one test sequence before the EEG implantation procedure, followed by another two sequences before administration of sarin or saline. No behavioural testing was conducted for one month after surgery or for one week periods between test sequences thereafter. Eight subjects received saline injections and nine received sarin. Groups were balanced on the basis of training performance as well as for other factors, such as gender, as far as possible.

Acetylcholinesterase Determination: Acetylcholinesterase activity in whole blood and plasma was determined by the method of Ellman et al (1961). Three control samples were obtained, one sample taken 3 hours following administration of sarin or saline and subsequent samples taken at approximately monthly intervals.

DATA HANDLING AND ANALYSIS

EEG:The approaches to EEG sampling and analysis have been reported elsewhere (Pearce et al 1998). EEG results were calculated as amplitude (μV) per Hz in the frequency bands delta (1-3.5 Hz), theta (3.5-7.5 Hz), alpha (7.5-13 Hz), beta 1 (13-22 Hz), beta 2 (22-40 Hz), beta (13-40 Hz) and total amplitude. Median frequency was also calculated. In order to make between animal comparisons as well as within animal comparisons, all data were normalised such that post dose data were calculated as a change from a mean of at least 10 recordings made pre-dose. Mixed model analyses of variance, including time as a linear covariate, were used to analyse the above 8 outcomes separately and a $\log_{10}$ transformation of the data was used to satisfy assumptions of normality (the Watson statistic) and equal variances (Bartletts test).

*Behaviour:*Mixed model analyses of variance with five fixed factors were used to compare the effects of Stimulus type (shape or line), Stage (SD,CD,IDS,EDS), Reversal, Period and Dose on the number of errors made in achieving criterion at each stage. Period was defined as period 1 (averages of sequences 4-6), period 2 (sequences 7-9) and period 3 (sequences 10-12), each expressed relative to the average of baseline sequences 1-3. The presence of any interaction between any of these effects was considered. Normality assumptions were checked using the Watson statistic and Bartlett's test was used to check for equal variances. As the residuals from the analysis of total number of errors data violated the above assumptions, a $\log_e$ (No. of errors + 1) transformation was used. Post-hoc analyses of significant effects was carried out using appropriate contrasts which involved investigating ratio of ratios.

RESULTS

EEG:Details of statistical interactions are given in Table 1. Figure 1 displays the mean amplitude per Hz for frequency bands theta, alpha, beta 1 and beta 2, expressed as a percentage change from pre-exposure values.

No significant changes between sarin and control groups as a pattern over time (dose × time interaction) in either theta ($p=0.65$) or alpha ($p=0.22$) frequency bands were noted, although there was a divergence between control and treated animals after 12 months recording in the latter.

In the beta 1 frequency band, there was no change in treated animals despite a non significant fall in amplitude in control animals (dose × time, $p=0.22$).

In the beta 2 frequency band, there was an increase in amplitude in treated animals when compared with both baseline values and control animals. Over time, the effect of sarin approached statistical significance ($p=0.07$). The estimated difference in slopes between control and sarin subjects was found to be 0.005 (confidence interval −0.8%, 1.8%).

representing an extra increase in beta 2 of 0.5% per month in sarin subjects. However, within the group of treated animals, amplitude increased by over 40% in one animal. If this animal was excluded from the results analysis, the dose × time interaction was found to be p=0.13 and the difference in slope of 0.08% per month (confidence interval −1.2%, 1.4%).

Neither delta, total amplitude nor median frequency parameters showed any differences approaching statistical significance. No significant differences between subjects assigned to respond to shapes and those assigned to lines (stimulus type) arose due to sarin administration.

Behaviour: The long-term performance profile obtained was comparable for the control and dose groups. Figure 2 illustrates the changes in the number of errors made in reaching criterion at each stage of each sequence corrected to a mean of the three sequences performed pre sarin or saline administration. Results of the behaviour analysis of sarin effects are shown in Table 2.

Three significant three way interactions were found for type×dose×stage ($p = 0.015$), type×reversal×stage ($p < 0.001$) and type×stage×period ($p < 0.001$). The three way interaction involving dose was investigated further, results of which are shown in Table 3, as this analysis would encompass factors seen in the two way interactions. The four-way interaction was not analysed further as the important aspects were considered in evaluation of the three way interactions.

On one component of the test, the first compound discrimination (CD), the sarin treated groups of animals performed significantly better than control animals for shapes, but for lines they were similar ($p<0.001$) (see Table 4). In addition, superior performance of the sarin treated group was seen at the simple discrimination stage (SD).

Acetylcholinesterase activity: Three hours following sarin administration mean erythrocyte cholinesterase inhibition was 51.3% (range 36.4 - 67.1%) and this returned to baseline levels 4-45 weeks later. When average cholinesterase activity three months after dosing was compared with average pre-dosing controls no significant difference was seen ($p=0.50$) so, on average, levels had returned to baseline by three months. No changes over time were seen in control subjects.

DISCUSSION

A single low dose of the organophosphorous compound sarin, leading to acetylcholinesterase inhibition of 51%, produced no significant changes in either EEG, as measured by radiotelemetry, and no decrement in cognitive behaviour, as measured by an attentional set-shifting task, in the common marmoset.

This lack of effect on EEG is inconsistent with previous studies (e.g. Burchfiel et al 1976) and unpublished work at CBD which showed small changes in the beta 2 frequency band in a limited number of rhesus monkeys 7-9 months following a comparable dose of sarin (2.5μg.kg^{-1}). There are significant methodological differences between these earlier rhesus monkey studies and the present marmoset study. In the present study, no anaesthesia or restraint was used immediately prior to EEG monitoring. Using this alternative approach, a noteworthy consistency in EEG within subjects was seen over time, possibly because of improved consistency of data capture conditions as EEG was recorded whilst the animals were responding to the behavioural test. Additionally, no loss of signal quality occurred over time. This illustrates the acceptability of this method of EEG collection and its ability to provide a good baseline for comparison with data obtained following pharmacological intervention. Administration of sarin did not result in a significant effect on any of the EEG measures recorded here. However there was a trend towards significance in the beta 2 band ($p=0.07$) which is worthy of note because it is in the same frequency range and direction that changes were seen in previous studies. The statistical analysis revealed that this trend was due to one outlying subject showing a larger progressive increase in beta 2 activity over the course of the study, which could be indicative of a threshold for effect which was reached in this subject alone. Additionally, visual inspection of the data initially indicated a downward trend in the beta 1 band for the control group, which was not seen in the exposed subjects. Again a single outlying subject accounted for this trend. There was no evidence of a behavioural change in these individuals. It does, however, raise the possibility of the existence of a species specific threshold for effect. This could be addressed in future studies involving a larger dose range.

The marmosets were successfully trained to perform an attentional set-shifting paradigm in their home cages. Analysis of behavioural performance over the 12-15 month period following sarin administration revealed no effects on any aspect of the multistage sequence. The behavioural sequence employed throughout the study was used because it contained elements which would be expected, on the basis of previous work, to be sensitive to detrimental effects caused by cholinesterase inhibitors. For example, Roberts et al (1992) showed a decrement in discrimination reversal performance following cholinergic lesions. Other elements in the test have been shown to be sensitive to other

manipulations e.g. dopaminergic lesions affect intradimensional versus extradimensional shift performance with comparison of these two stages, giving a measure of set-shifting performance (Roberts et al 1994).

Results from the paradigm presented do not indicate any detrimental effects associated with the administration of sarin at any point post administration. The significant dose interaction was seen to reflect an improvement in the CD stage in 'shape' subjects at a single time point. The significance of this improvement is unclear. Most of the other interactions seen in the analysis of behavioural performance revealed by the ANOVA were predicted at the start of the study. Reversal elements were consistently found to be more difficult than the preceding discriminations. On the set-shifting element of the sequence, 'shape' subjects found the intradimensional shift easier than the extradimensional shift, a result consistent with findings reported elsewhere (e.g. Roberts et al 1988). This pattern of performance is consistent with the predictions of two stage theories of attention and may be taken as being indicative of learning set formation. It has been suggested that the ID and ED elements of this type of behavioural test are of equal complexity but require different abilities (Roberts & Sahakian 1993). This contrasts with the performance of subjects assigned to the line group which found the ED shift easier than the ID shift. This effect has been reported previously (Crofts et al 1996) and may represent interaction between the saliency of the dimensions, the test sequence employed and the learning criterion. Thus, for this paradigm, in future studies it will be necessary to treat the groups assigned to the line and shape dimensions separately. Over the course of the experiment there was a trend towards improved performance of the behavioural sequence in all groups. Overall, there was no deleterious effect of sarin on either the shape or the line group, on any element of the test sequence or on the rate of improvement in performance over the year.

This study offers further validation of the approaches and techniques employed. Home cage behavioural testing of marmosets on tests from CANTAB has previously been shown to be viable (Crofts et al 1995, 1996, Pearce et al 1998) and collection of behavioural data over a period of more than 24 months illustrates that this method is suitable for the sort of long term study reported here. Additionally, the collection of EEG by telemetry has illustrated that electrophysiological data can also be collected over a long period without adverse effects due to the presence of the transmitter. Use of the telemetry techniques also enables common marmosets to be used in studies where eletrophysiological measures are desirable. The use of a small implantable transmitter means that this is a much more attractive option than those previously available and may mean that it could be possible to employ this small, new world primate in studies where previously larger species, for example, the old world rhesus macaque, may have been used. Furthermore, the opportunity for remote electrophysiological monitoring in the recent study has also enabled the characterisation of sleep patterns. These data which are currently undergoing analysis, may further elucidate the sequelae of exposure to low doses of OP compounds.

The results of this study illustrate no significant effects on EEG and no deleterious effects on cognitive performance following a dose of sarin that inhibited erythrocyte cholinesterase by 51%. The degree to which peripheral measures of cholinesterase activity reflect central enzyme activity is not clear but in vivo central cholinesterase measurement techniques are limited. Suggestions of a 'threshold for effect' mean further studies are required to investigate the dose-response for sarin and other OP compounds.

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FIGURES AND TABLES

Figure 1. Changes in EEG frequency bands from baseline over time

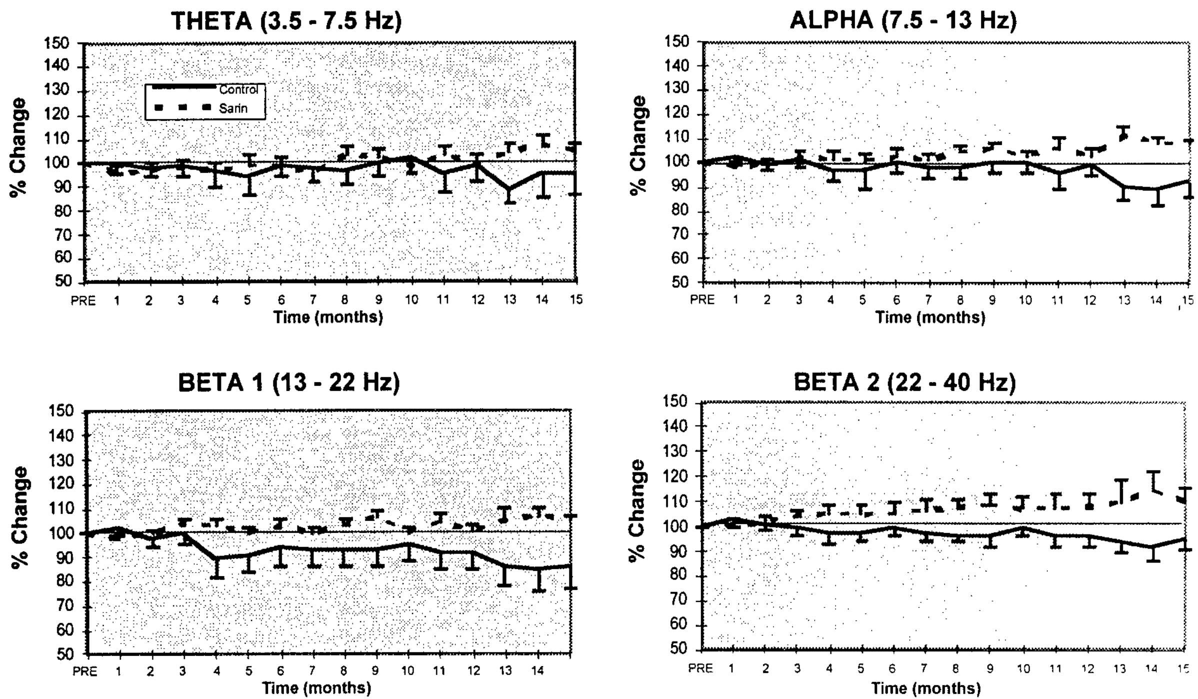

Figure 2: Changes in percent mean errors made in reaching criterion on each behavioural sequence presented

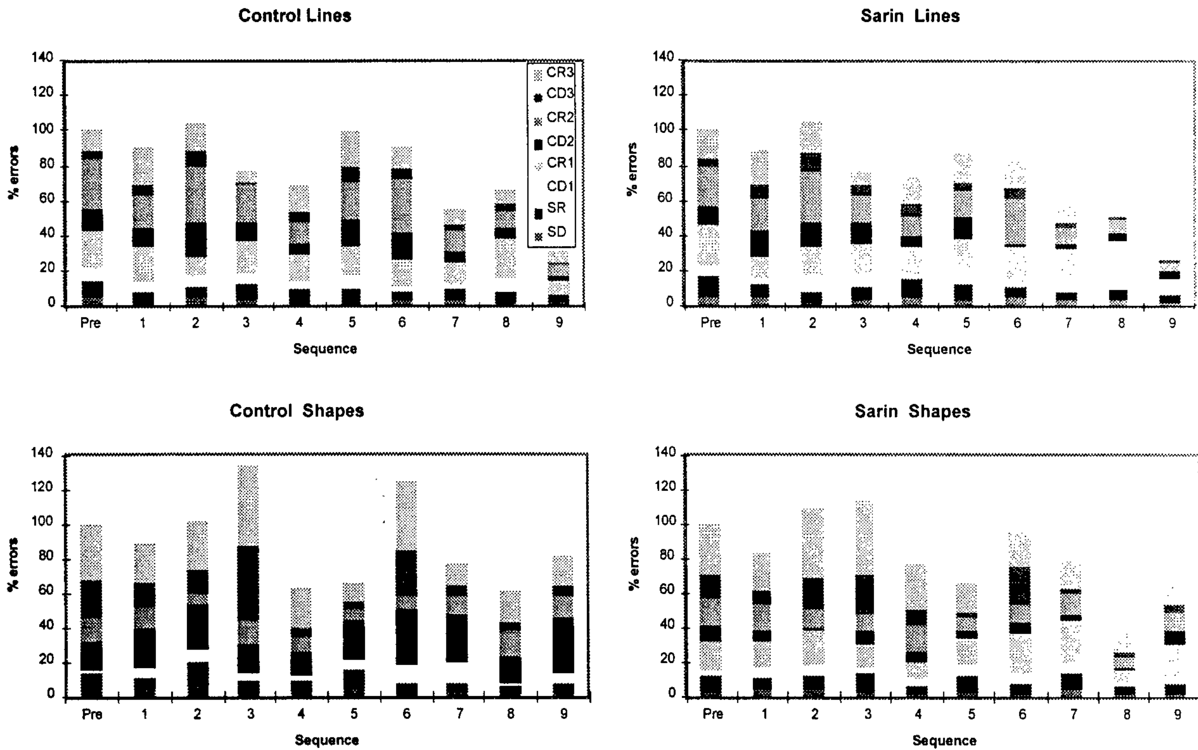

Table 1: Results from EEG ANOVA

Effect	p values							
	Median	Delta	Theta	Alpha	Beta 1	Beta 2	Beta	Total Amp
Stimulus type	0.21	0.11	0.32	0.58	0.53	0.53	0.53	0.18
Time	0.17	0.32	0.44	0.40	0.75	0.65	1.00	0.53
Dose	0.21	0.29	0.75	0.34	0.11	0.06	0.07	0.29
Type × Time	0.44	0.75	1.00	0.65	0.58	0.32	0.50	0.75
Type × Dose	0.58	0.75	0.75	1.00	0.44	0.58	0.65	1.00
Dose × Time	0.25	0.21	0.65	0.22	0.22	0.07	0.16	0.37
Type × Dose × Time	0.37	0.75	0.75	0.37	0.25	0.50	0.27	0.58

Table 2: Results of ANOVA analysis of behaviour

Effect	p value
type	0.65
dose	0.75
rev	0.44
stage	<0.001
period	<0.001
type*dose	0.29
type*rev	0.13
dose*rev	0.65
type*stage	<0.001
dose*stage	0.08
rev*stage	0.044
type*period	0.037
dose*period	0.067
rev*period	0.047
stage*period	<0.001
type*dose*rev	0.65
type*dose*stage	0.015
type*rev*stage	<0.001
dose*rev*stage	0.87
type*dose*period	0.65
type*rev*period	0.64
dose*rev*period	0.99
type*stage*period	<0.001
dose*stage*period	0.66
rev*stage*period	0.22
type*dose*rev*stage	0.61
type*dose*rev*period	0.86
type*dose*stage*period	0.05
type*rev*stage*period	0.46
dose*rev*stage*period	0.99
type*dose*rev*stage*period	0.72

Table 3: Post-hoc analysis of dose effects by stage (ratio of stimulus type (shape : line) for sarin treated animals relative to control animals)

Stage	ratio	95% Confidence Intervals	p
SD	0.69	0.46, 1.04	0.07
CD	0.47	0.31, 0.69	0.001
ID	0.74	0.49, 1.10	0.14
ED	1.21	0.81, 1.81	0.36

Table 4: Comparison of the mean errors on CD1 made by control and sarin dosed 'shape' subjects (untransformed data)

	Baseline	Post dose
Control mean	6.083	26.75
SEM	1.46	4.51
Dose mean	16.133	23.211
SEM	9.14	2.87

26. SEARCH FOR NEW NEUROPROTECTIVE DRUGS AGAINST SOMAN-INDUCED CENTRAL NEUROPATHOLOGY: ANTIOXIDANTS

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ABSTRACT

Injecting rodents with soman (GD), a potent organophosphate nerve agent, quickly induces seizures evolving into status epilepticus and death at lethal dosage. Central neuropathology is especially prominent in some structures such as in the limbic and thalamic areas. Glutamatergic neurotransmission is a key player in the onset of the neurological insult. Oxygen free radicals are most probably involved although evidence for their production and/or toxic role during soman intoxication is still scarce. 21-aminosteroid (lazaroids) are potent inhibitors of iron-catalyzed lipid peroxidation in neural tissue and have been developed for the acute treatment of central nervous system injury and ischemia. We focused on two typical representatives of this family, U-74389G and U-83836E, and tested their neuroprotective properties in mice pretreated with HI-6 (50 mg/kg, i.p.) and challenged with a convulsant dose of soman (172 µg/kg). In these preliminary studies, pretreatment or treatment with these molecules could not prevent seizures. Pretreatment or treatment with U-74389G (5 and 10 mg/kg) or U-83836E (14 mg/kg) led to inconsistent neuroprotection in hippocampus following soman intoxication. Similarly U-74389G could not prevent or reverse the increase of [^{3}H]PK11195 binding, a ligand of peripheral benzodiazepine receptor, used as a neuropathological index. U-83836E by itself increased the binding of this ligand leading to a difficulty in assessing its potential benefit. Conversely, U-74389G (5 and 10 mg/kg) tended to reduce malondialdehyde levels in cortices of intoxicated animals suggesting a dissociation between neuroprotection and antioxidant properties. Further studies are in progress.

INTRODUCTION

The highly toxic organophosphorous nerve agent soman (pinacolyl methylphosphonofluoridate) is a potent irreversible inhibitor of the enzyme acetylcholinesterase (AChE). Following experimental exposure, rodents suffer limbic seizures of quick onset, rapidly generalized and evolving into status epilepticus. The build-up of acetylcholine concentrations at central synapses due to the inhibition of AChE is essential to seizure onset [9,16]. A secondary increase of other neurotransmitters, especially excitatory amino acids such as L-glutamate (Glu), is a key process in neuropathology development. Glu is well known to be involved in several central nervous system pathologies [5,20]. Histopathological examination of rodent brains, hours after injection of soman, revealed severe neuronal damage in different regions of the brain, especially the hippocampus, piriform cortex, neocortex and thalamus [e.g. 13,15]. Even though the role of Glu appeared prominent, as evidenced by the neuroprotection afforded by ionotropic glutamate receptors [10-12], little is known about the precise biochemical mechanisms that lead to neuronal death. It is believed that Glu mediates neuronal injury via several mechanisms and that reactive oxygen species (ROS) production is involved in the neurotoxicity [3,8]. Evidence of oxidative stress during soman intoxication is scarce [7,14]. However, a recent study pointed out an increased level of lipidoperoxidation products in rat brain as early as 30 min. after the injection of a convulsing dose in areas where lesions are described (e.g. hippocampus and thalamus) [7]. This working hypothesis led us to investigate the potential benefits of the administration of two antioxidants from the 21-aminosteroid family, U-74389G and U-83836E. This family of non-glucocorticoid compounds has been developed as cytoprotective lipid peroxidation inhibitor for the treatment of traumatic and ischemic central nervous system injuries [6]. Some of them have also been tested in seizure experimental models [1,2]. The compound's ability to inhibit lipid peroxidation resides in the combination of a chemical antioxidant action and a decrease in the fluidity of membrane phospholipids [6].

In the present study, our preliminary results suggest that neither U-74389G nor U-83836E appear to significantly prevent hippocampal neuronal damage (cresyl violet staining) or glial reaction (ω3 binding studies) induced by the injection of a convulsant dose of soman. Conversely, U-74389G tended to decrease lipidoperoxidation in cortex.

METHODS

Chemicals

Soman (97,9 % pure, CPG) was obtained from the Centre d'Etudes du Bouchet (France). [^{3}H]PK11195 (specific activity = 86 Ci/mmol) was obtained from NEN™ Life Science, Inc. (France). The 21-aminosteroids U-74389G and U-83836E were obtained from Biomol Research Laboratories (TEBU, France). U-74389G was first dissolved in CS-4 sterile vehicle and then loaded into a pre-formed emulsion (i.e., Lipoven ®) and the pH adjusted to ca. 7 with 100 mM pH 7 phosphate buffer. U-83836E was dissolved at the required concentration in normal saline. MDA was prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane (ICN Biomedicals, Inc., France) at 95°C for 10 min.

Animals

Adult male mice, weighing 25-30 g were obtained from Elevage Janvier France. Animals were maintained on a 12h/12h light-dark cycle and given food and water *ad libitum*. The experimental protocol and procedures used meet the French and European community guidelines and have been approved by the Animal Care Committee (CRSSA).

Intoxication and determination of the LD_{50} of soman in the model with HI-6 pretreatment

The oxime HI-6 (50 mg/kg, i.p.) was given to the animals 5 min. prior to soman challenge with a convulsing dose (172 µg/kg, s.c.). LD_{50} was determined by the moving-average interpolation method applied to the number of dead mice 24 hr after soman challenge [19,21].

Determination of the neuroprotective activity of U-74389G and U-83836E

Neuroprotection afforded by the drugs was assessed by different means:

• Measurement of ω3 site densities in hippocampus of soman-treated mice ([10] with modifications)

48 h after soman treatment, mice were decapitated and the hippocampi dissected, frozen and stored at -80°C until assay. Hippocampi were weighed, Polytron homogenized in 20 % (w/v) of a 120 mM NaCl, 50 mM Tris-HCl (pH 7.4) buffer. Aliquots of 25 µL were used for protein determination using the method of Lowry et al. Aliquots of 100 µL of homogenate (ca. 500 µg of protein) were incubated with 2 nM [^{3}H]PK11195 in 1 mL (final volume) of Tris-saline buffer for 30 min. at +25°C. The incubate was decanted by vacuum filtration through GFB filters; the filters were rinsed 3 times with 3.5 mL of cold Tris-saline buffer and the bound radioactivity was determined by liquid scintillation spectrometry. Non-specific binding was determined using 1 µM PK11195. Binding was measured in duplicate.

• Histological analysis by cresyl violet staining

48 hours after soman intoxication, mice were decapitated and their brain rapidly removed. They were immediately frozen in dry ice cooled isopentane. Serial coronal brain tissue sections (10 µm) were obtained with a cryostat. A classic cresyl violet staining procedure was then applied. Presence or absence of hippocampal lesions was used as a marker for neuroprotection.

Measurement of the antioxidant effect of U-74389G

The animals were killed by decapitation 48 h after the injection of soman and the brain rapidly dissected on ice. For this preliminary study, it was divided into two parts, cortex and the remaining portion. They were weighed and homogenized using a Teflon pestle in ice-cold 0,2 M phosphate buffer pH 7.4 (10% w/v). Aliquots of 500 µL of homogenates were diluted 1:2 with distilled water and 50 µL of a 2% Butylated hydroxytoluene solution in ethanol were added to prevent further oxidation. The homogenates were frozen and kept at -80°C. The day of the experiment, they were thawed and an aliquot was treated with 50% trichloracetic acid, centrifuged at 2000 g for 10 min. at +4°C. Malondialdehyde (MDA) and other end products derived from peroxidation of polyunsaturated fatty acids and related esters were assayed as thiobarbituric acid reactive substances (TBARS). Briefly, an aliquot of the supernatant was collected and mixed with 0.67% thiobarbituric acid (TBA) and incubated for 10 min. at +95°C. TBARS were measured by spectrofluorimetry (λexc 532 nm, λem 553 nm).

Data analysis and statistics

Data of MDA (TBARS) content and [^{3}H]PK11195 binding values are expressed as mean ± SEM. All statistical analyses (for groups with at least n=5) were performed using nonparametric tests (Kruskal-Wallis test followed by post-hoc Mann-Whitney test using the corrective Bonferroni's procedure).

RESULTS AND DISCUSSION

Pretreatment with HI-6 increases the survivability of the animals and allows higher soman challenges. In this model, LD_{50} was 278 µg/kg (95% confidence limits: 248-311 µg/kg), higher than LD_{50} without HI-6 (ca. 110 µg/kg). This model is derived from the rat model previously described by Shih [17]. All animals injected with soman (172 µg/kg, s.c.) developed classic signs of nerve agent intoxication including spontaneous tremor, limbic seizures and hypersecretion. The first visually observed limbic-type movements appeared on average 4.2 ± 0.5 min. (mean ± SEM, n=18) after soman injection. Most survived at least 48 hr. At 24 and 48 hr, histological examination of

mouse brains often revealed obvious damage to hippocampus, especially in CA1 and CA3 subsectors (Figure 1). Cresyl violet staining does not easily reveal lesions in other parts of the brain (e.g. thalamic area).

Neither U-74389G nor U-83836E, injected as pretreatment 30 min. prior to soman challenge or as treatment 15 min. post-challenge, interfered with seizure onset and status epilepticus. This is not surprising given the neurochemical basis of soman-induced seizures and consistent with previous studies using other convulsant agents [1,2]. Pretreatment with U-74389G (5 and 10 mg/kg) or U-83836E (14 mg/kg) led to inconsistent neuroprotection in hippocampus following soman intoxication (Figure 2). Owing to the variability of hippocampal lesions in soman-intoxicated mice compared to other damaged brain structures, it is therefore difficult to clearly conclude. Other histological analyses are currently being conducted. Increase of the binding of [^{3}H]PK11195, a ligand of the peripheral benzodiazepine receptors [4], is considered to be a suitable marker of soman-induced brain lesions [10]. U-74389G could not prevent or reverse the increase of [^{3}H]PK11195 binding. In the case of U-83836E we found that the drug itself could increase [^{3}H]PK11195 binding thus rendering a more complex analysis of the results. The results obtained with both lazaroids are thus consistent with the histological findings and suggest a limited effectiveness (Figure 3). Antioxidant properties of U-74389G were evaluated in cortex and our first results are in favor of a reduction of TBARS following administration of this lazaroid (Figure 4). This suggests a dissociation between neuroprotection and antioxidant properties of the molecule. Such a dissociation has been reported for U-83836E in vitro [18].

CONCLUSIONS

Neither U-74389G nor U-83836E appears to significantly prevent hippocampal neuronal damage or glial reaction induced by the injection of a convulsant dose of soman despite a tendency to decrease lipidoperoxidation (studied with U-74389G). Studies are in progress to confirm these findings especially the relative dissociation between the antioxidant and neuroprotective properties of U-74389G.

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KEYWORDS

soman, organophosphorous compound, seizures, neuroprotection, 21-aminosteroid

FIGURES AND TABLES

Figure 1 - Light microscopy pictures showing soman-induced hippocampal neuronal loss or modifications. Brain tissue coronal sections (10 µm) from a control mouse (A) and those injected with soman (172 µg/kg) after 24 hr (B) and 48 hr (C). The loss of neurons in the pyramidal cell layer of the CA1 hippocampal area is obvious. Cresyl violet acetate staining. Scale bar = 400 µm.

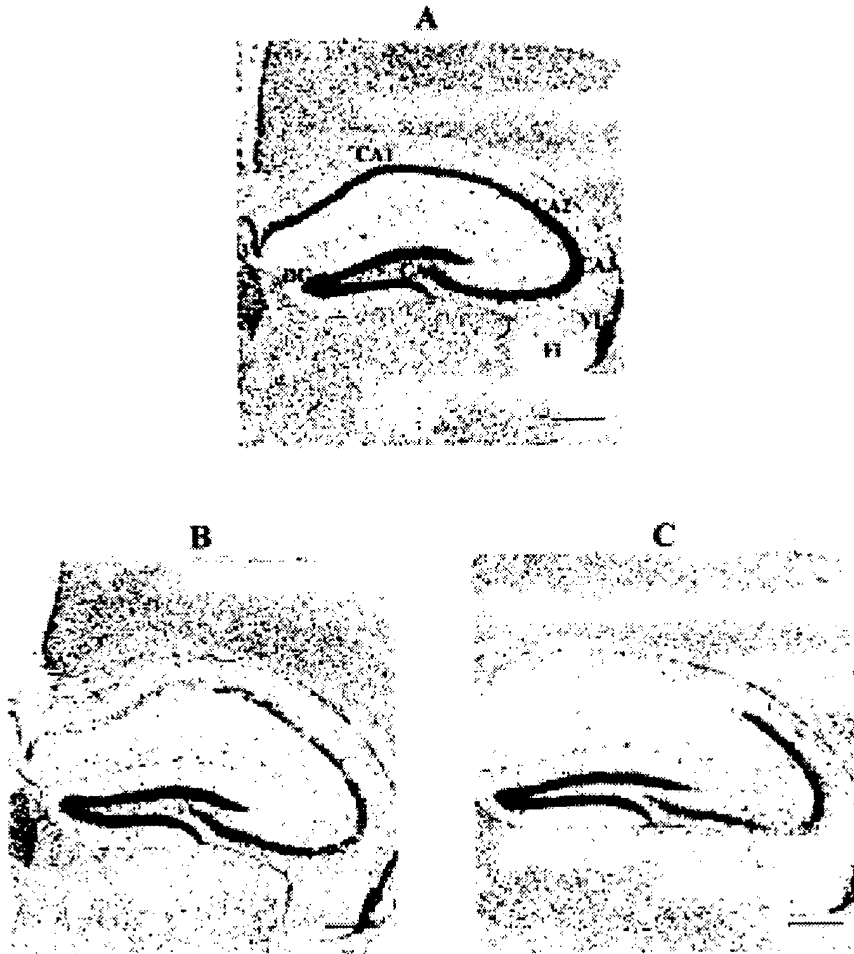


Figure 2 - Light microscopy pictures showing soman-induced CA1 pyramidal cell loss or modifications 48 hr after the challenge and the lack of clear improvement by 21-aminosteroid pretreatment. Brain tissue coronal sections (10 µm) from a control mouse (B), and those injected with soman (172 µg/kg) alone (A) or receiving additionally, 30 min. previously, an intraperitoneal injection of U-74389G (10 mg/kg) (C) or U-83836E (14 mg/kg) (D). Note the loss of neurons in the pyramidal cell layer of the CA1 hippocampal area (between arrows). Cresyl violet acetate staining. Scale bar = 100 µm.

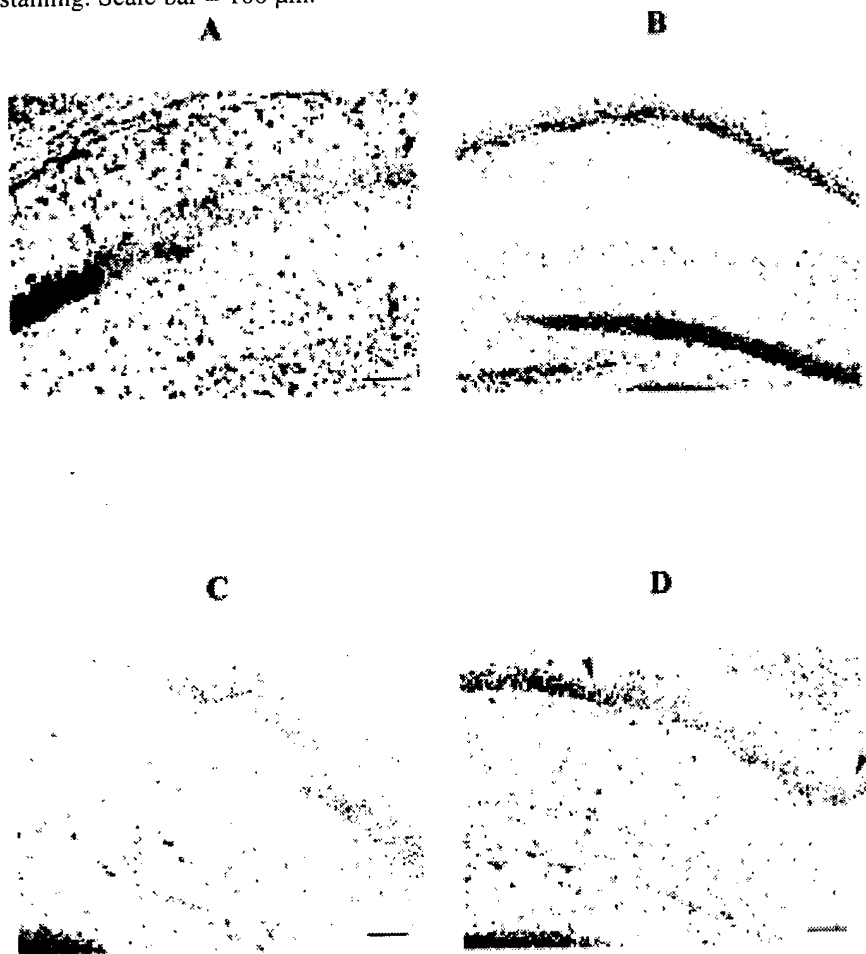


Figure 3 - [^{3}H]PK11195 specific binding on mouse hippocampal homogenates 48 hr after the indicated treatment. Control groups U-74389G and U-83836E only received the drug (10 mg/kg). Pretreatment and treatment are injected 30 min. prior to and 15 min. after the soman challenge (172 µg/kg) respectively. Data are presented as mean ± SEM of n separate determinations. For each of the molecules, experimental groups are compared using the Kruskal-Wallis nonparametric test followed by post-hoc pairwise comparisons with the Mann-Whitney test corrected by the Bonferroni's procedure (k=10). Comparison to control (saline) ** p < 0,001 (α 1%), *** p<0,0001 (α 1%).

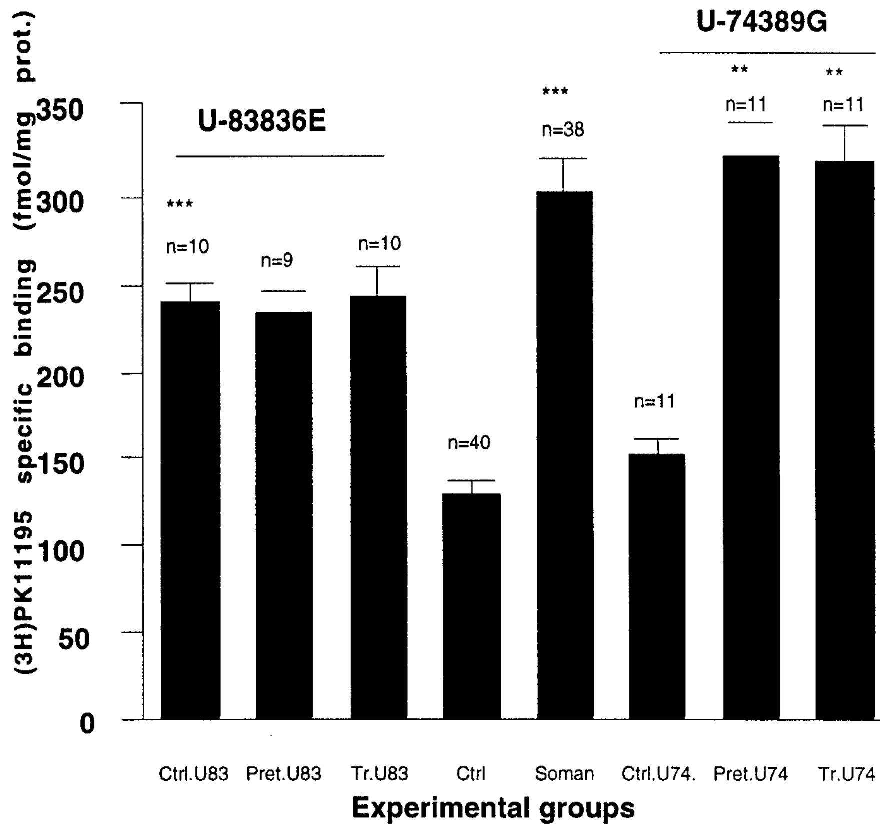
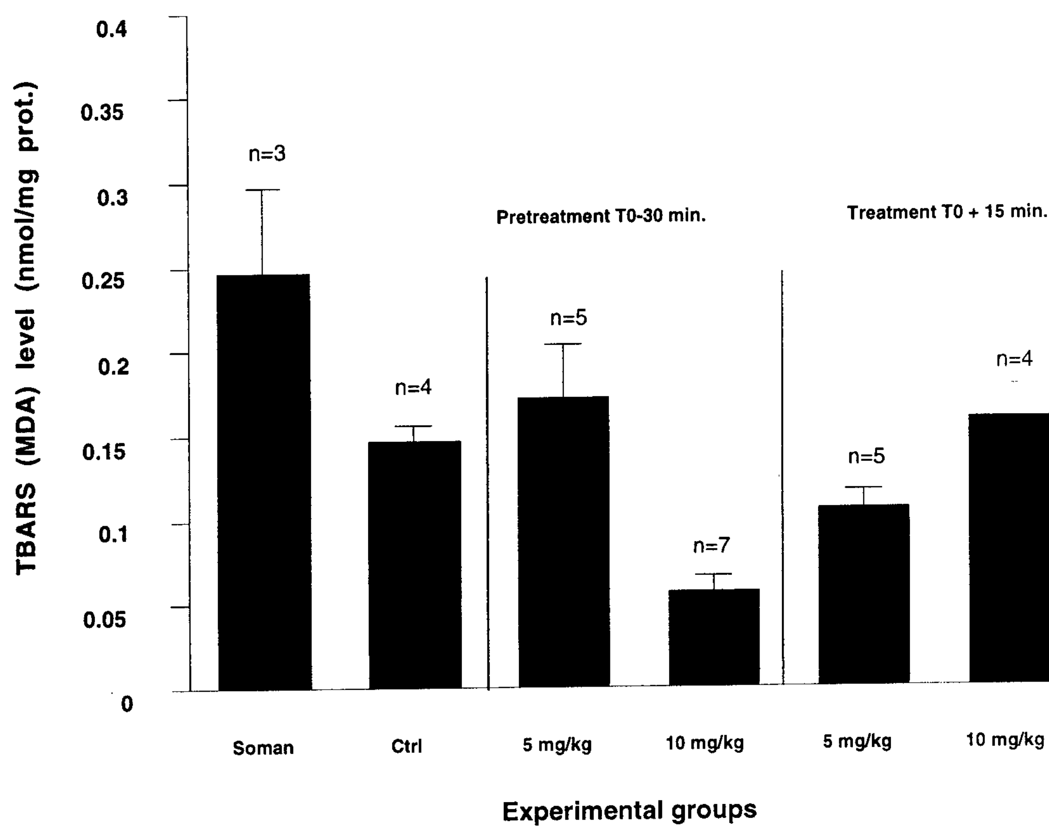


Figure 4 - Effect of U-74389G on TBARS (MDA) level in mouse neocortex 48 hr after a soman challenge (172 µg/kg). Results are presented as mean ± SEM of n separate determinations.



27. HUMAN PARAOXONASE AS A CATALYTIC SCAVENGER AGAINST CW ORGANOPHOSPHATES.

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During the past 10 years, various OP hydrolases have been explored as potential antidotes against OP poisoning. Hence, it has recently been demonstrated that the administration of the bacterial phosphotriesterase (PTE) to mice could improve the classical treatment of sarin poisoning. Furthermore, it is well established that the presence of endogenous scavengers in plasma plays an important role in determining the susceptibility to OPs. We currently study human paraoxonase (PON1, E.C. 3.1.8.1), a naturally-occurring serum catalytic scavenger. Human PON1 is a HDL-associated enzyme which exhibits a calcium-dependent organophosphatase activity toward various substrates, including the nerve agents sarin, soman and VX. Our main objective is to modify human PON1 to increase its catalytic efficiency by a 100-fold factor toward sarin, soman and VX. Rational design of PON1 mutants with an improved catalytic efficiency toward OPs first requires to identify the PON1 active site components and to solve its 3D structure. Recombinant human PON1 wild-type and mutants can be expressed in CHO cells. Another important issue is to produce human PON1 as a soluble and active enzyme in Escherichia coli.

Keywords: paraoxonase, organophosphate, scavenger, engineering

28. ON THE OLD MILITARY "HERITAGE" AND CHEMICAL WASTES

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ABSTRACT
Uncontrolled waste sites, including waste storage and treatment facilities, former industrial and agricultural properties and military facilities may produce health or environmental effects when chemicals released from these site disperse into air, into water, and on and under the ground. The potential impact of disasters is most marked in developing countries. Developing countries are not able to take appropriate measures for preventing accidents, treating victims, and aiding environmental recovery. This problem is much more difficult in developing countries due to lack of technical and financial resources. Many officials responsible for the management of chemicals lack knowledge of the hazard of chemicals and how to diagnose and treat unusual chemical poisoning. In addition, which is so important, analytical facilities to detect and identify chemicals causing poisoning are not available. This limits the ability to choose appropriate antidotes. The present paper extends the review of current information on environmental threats to human health from old military facilities and abandoned chemical waste sites in Georgia and estimates the position to organize work to decontaminate toxic chemical waste and to prevent its negative impact on the environment and human health.

INTRODUCTION
It is well known that uncontrolled waste sites, including waste storage and treatment facilities, former industrial and agricultural properties and military facilities can contribute to health and environmental problems. Particularly, these sites are a concern to developing countries with economics in transition. We conducted this survey at the time when Georgia is undergoing serious domestic economic and social transformation. The rapid negative changes of recent years created many problems in developing the national infrastructure for managing chemicals. It might be said that almost all systems to control chemicals there have been destroyed and disorganized.

People living within one kilometer of hazardous waste disposal sites are at risk of exposure to chemical toxicants released from these sites into the air, groundwater, surface water, and surrounding communities. Children are uniquely vulnerable to these toxicants. The substances most commonly released into environmental media from uncontrolled hazardous waste sites are heavy metals and organic solvents: lead (59% of sites), trichlorethylene (53%), chromium (47%), benzene (46%) and arsenic (45%) (1,2).

There is a growing awareness that inadequately controlled chemicals can affect central nervous, reproductive, gastrointestinal systems and cause hematological, mutagenic and carcinogenic effects. They can also influence the activity of the immune system by either augmenting or suppressing its function (3).

The purpose of this survey was to evaluate the health risk of exposure to hazardous waste disposal sites and estimate positions needed to organize work to decontaminate toxic chemical wastes and to prevent their negative affects on environment and human health.

METHODS
Data from different organizations and interested parties regarding uncontrolled waste sites were analyzed with respect as to how toxic chemicals released from these sites may impact on human health. A retrospective review was conducted on poison exposure patients from 12/1/1999 - 15/1/2000. Data regarding the place of poisoning, outcome of cases, and site of treatment were collected.

RESULTS AND DISCUSSIONS
The results of this study indicate that the majority of uncontrolled waste sites in Georgia are waste storage and treatment facilities or former industrial and agricultural properties. Many of these properties have been abandoned. Before disintegration of the SU there were situated various military bases of the former Soviet Union (SU) which were strongly guarded and secret. At present, many of the USSR Armed Forces have abandoned Georgia. Almost all their previous bases were ruined and left uncontrolled.

At present, about 26 tons of "Rocket fuel"(mixture of TG-2, technical xcilidin and triethylamine) and 480 tons of "Mélange" (AK-20k, nitric acid 73%, nitrogen oxides) are left at the former military airport in Meria. 170 tons of "Mélange" is also left in the village of Tshaladidi (near Poti) and 60 tons in Soganlugi (near Tbilisi). 18 tons of old

chloropicrin has been left in pesticide storehouses in Gardabani. There are also many pesticides and fertilizers in storehouses and abandoned pesticide formulating facilities that need to be inventoried.

From a toxicological point of view, the old uncontrolled military "heritage", such as liquid fuel of old rockets (cyclonite and triethylamine), large amounts of chloropicrin and other unknown chemicals are hazardous to a peaceful population. Many of these same chemicals can be used in industrial processes and, in some cases, pesticide manufacturing. There is evidence suggesting that peasants in Kakheti applied unknown chemicals (from military waste) in their vineyard. As a result, the vineyards were fully brined and people and cattle were poisoned.

In over one year of conducting this survey, 127 poison exposure cases were registered. Acute poisonings occurred in 105 (82.7%) of these cases, while 22 (17.3%) were chronic poisoning. It should be taken into account that these data are not complete, because many cases of poisoning are not registered. Because the existing analytical equipment in the analytical laboratories is limited, biological monitoring to identify the chemicals in many of the poisonings was not available. The analytical methods used are in accordance with former USSR manuals.

At present most pesticide and fertilizer storehouses are in very bad condition. And they do not answer to environmental and sanitary demands. Transportation, usage, distribution and storage of chemicals are not regulated. Hazardous chemicals are on sale everywhere - in the market, on the roads, in boxes with food products, often without labeling. It is impossible to control chemical residues in food. Georgia does not have a special waste disposal system. Due to lack of financial resources, it is difficult to take appropriate measures for utilization, segregation, recovering and recycling of waste. In most cases, there is a complete absence of routine monitoring due to old analytical laboratory equipment, absence of reagents, and standards for environmental and biological monitoring.

In accordance with the National Fire Protection Association (4), hazardous materials are defined as "any substance that causes or may cause adverse effects on the health or safety of employees, the general public, or the environment and/or any biological agent and other diseases -causing agent and /or waste or combination of wastes". In this regard, toxic waste sites can contribute to health and environmental problems. And if the appropriate measures are not taken in time, inhabitants of our society may be became victims because of inadvertent releases of hazardous substances.

CONCLUSIONS

Huge social, political, environmental and health problems still confront Georgia. There are no resources to provide decontamination of toxic chemicals left without any control at the old military bases and industrial and agricultural facilities.

The situation at the laboratories also reflects the political and economic situation. Due to lack of funding, all of those laboratories are destroyed. Otherwise, the absence of reagents, glassware and the presence of old and unserviceable equipment meant that no monitoring had taken place since 1990. Often there is no electricity (especially in wintertime) and the water supply is cut off at intervals. Air conditioning is not available in laboratories. The ventilation system in most laboratories does not work and is in need of repair. There is a need to improve the general laboratory facilities of the laboratories. It is recommended to provide laboratories with a basic package of laboratory equipment and consumables.

These problems cannot be solved without international assistance and implementing effective local, national and regional preventive initiatives of the government institutions and other entities. The results of this study will also need to be confirmed with larger study groups.

In order to protect human health against the harmful effects of hazardous uncontrolled chemical waste the following actions are recommended:

identify existing number of contaminated sites;
provide risk assessment of toxic waste sites;
clean up and redemption of hazardous waste sites across Georgia;
improve environmental and biological monitoring;
improve the national legal instruments related to chemical management;
improve the general laboratory facilities with major analytical and computing equipment; provide training to professionals;
study the adverse effects on human health of exposure to environmental toxicants associated with hazardous waste sites;
elaborate actions for fundraising and to stimulate domestic and outside financing.

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KEYWORDS
Hazardous waste, military facilities, industrial, agricultural properties, poisonings, environmental, biological, monitoring.

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29. TOXICOLOGICAL ASPECTS OF EVENTS IN TBILISI IN APRIL, 1989

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ABSTRACT
This paper is concerned with the tragic events of a toxicological disaster in Georgia in 1989, known as "9 April Tragedy", where militaries used unknown gases against peaceful demonstrators. Approximately 4000 people were poisoned, 16 died, 707 poisoned people were admitted to hospitals. The toxic agent that caused the poisoning was not identified in time to assist in treating the patients. Specific treatment of victims was delayed and no antidotes were used. Only international assistance and hard work of emergency physicians and clinical toxicologists were able to save some of the poisoned people. Lessons learned from this disaster have shown that it is important to have proper emergency plans, which include necessary actions based on toxicology. For this, emergency physicians need to acquire knowledge in the complex field of emergency toxicology. Identification of agents potentially causing toxicity is another critical point.

INTRODUCTION
The traditional forms of action in response to emergencies or toxicological disasters, originally consists mainly of immediate aid in the form of urgently needed drugs, medical equipment and other medical supplies. It is crucial, if not vital to quickly assess the existing situation and evaluate the risk of disaster. Emergency relief, disaster preparedness and management of disasters are the three main lines of action for the Emergency Department. The aim of this paper is to describe the tragic events of the toxicological disaster in Georgia on April 9, 1989, where militaries used unknown gases against peaceful demonstrators. Lessons learned from this disaster have shown that the Georgian toxicological service was not prepared for such events, and in the future, it must improve its emergency preparedness.

METHODS
Data were collected from the toxicological departments of main hospitals of Tbilisi, where poisoned patients were evaluated or treated. We compared the number and rate of victims of the above events: 1. brought to the toxicology department and not hospitalized, 2. hospitalized with mild, moderate and severe poisonings, average length of stay in hospital .

RESULTS
On April 9, 1989, in suppressing a peaceful demonstration in Tbilisi, toxic gases, later identified as the chemical warfare agents "Cheriomukcha" and "K-51", resulted in the death of 16 demonstrators and in more than 4.053 people requiring emergency medical evaluation. However, when the incident occurred and demonstrators were in need of proper medical attention, the militaries became an even more crucial issue. They insisted that the gas they had used was non-poisonous, that recovery was almost certain, and that there would be no long-term effects. At the same time, the militaries refused to provide either the composition of or toxicological information on the gases, on the nature of the injuries that could be caused from exposure, or the antidotes or specific treatments which needed to be prescribed. As toxicological departments of main hospitals of Tbilisi were not equipped with good analytical laboratories, there was no way to identify toxic substances in time. Later, it was estimated (B.Tchumburidze et al., 1990) by gas-chromatographic analyzes, that four types of "Cheriomukcha" were used and each of them contained different unknown chemicals. The major component was chloracetophenone. The composition of K-51 consisted of more than 30 compounds including C-S (45%). In addition, chloropicrin was discovered in samples of soil from the affected area.

P.Nishnianidze et al (1990) described 707 of the victims who were evaluated or treated in different hospitals in Tbilisi. The patient's condition, based on signs and symptoms, was classified as mild, moderate or severe. The clinical picture of "severe" cases resulted from acute chemical inhalation poisoning. Many of these patients were complicated with trauma. Among the 12 "severe" cases, nine were transferred to hospital unconscious, in a toxic coma. Physical examinations revealed mydriasis, tachycardia, multiple skin rashes, sclera irritation, hyperemia and different intensive hemorrhages. Of the latter, two sustained severe breath lesions and died on the second day after admission. In the majority of patients toxic bronchitis, toxic pneumonia and toxic pulmonary edema developed. Patients returning from coma were accompanied by delirium and hallucination.

Neurotoxic and irritant syndromes were detected in mild-moderate cases. Moderate cases were characterized by systemic signs and symptoms such as weakness, difficulty in breathing, short-term loss of consciousness, feeling of tightness in the throat, but did not require specifically mechanical ventilation. The symptoms of "mild" cases were headache, dizziness, cough, nausea, burning sensation of nose or eyes and lacrimination. All patients were very anxious for a long time, and sometimes had short-term memory loss. In the majority of patients, acute stress disorder was also diagnosed.

Clinical peculiarities of neurointoxication were studied by R.Shakarashvili et al. (1990). 142 patients intoxicated during events of April 9 were investigated. Besides routine clinical investigations EEC, EMC, CT, otoneurological, neurophatological and neuropsychological investigations were performed. Neurological symptoms of organic damage of nervous system were manifested mostly by following syndromes: corticopyramidal, brain system-vestibular, amnesiac, extrapyramidal (with tonic disturbances, hyperkinesis, convulsions), spinal-segmental, spinal-radicular, diencephal-hypotalamic, vegeto-ganglional, polyradiculoneurophatological, disautono-polyneurophatic syndrome. CV investigation showed hydrocephalous and progressing brain strophe. DMC indicated a demyelinating of peripheral nerves.

A.Lachkebiani et al. (1990) observed EEG changes of poisoned persons after 1 year of poisoning indicated increased neural activity and diffuse changes of cerebral electrogenesis, which testifies to the presence of nonspecific structures pathology. Neurological examination of poisoned with unknown gases people showed the presence of organic lesions of neural system.

At the time of publication, almost all surviving patients were classified as "full recoveries". However, patients of the "severe" and "moderate" categories still require surveillance, possible for years, to determine the presence or extent of possible adverse, long-term health effects.

CONCLUSIONS

The purpose of this survey is to show what can be done to be well prepared for toxicological disasters. As a result it is recommended that precautionary measures be taken by emergency departments, to include the establishment or alliance with a good analytical laboratory, equipped with high skilled personal. The staff in a Chemical Incident Response Services/ Toxicological Department needs to beware of the potential hazards mistakes in recognition can cause. Until a released chemical is properly identified and appropriate protection available, no handling should take place. Training needs to provide to professionals: emergency physicians need training in the complex field of emergency toxicology

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30. THE DETERMINATION OF HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE (ERY-ACHE) IN A MODIFIED ELLMAN ASSAY

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ABSTRACT

The colorimetric Ellman method is the most commonly used assay for the determination of human Ery-AChE in occupational health screening and therapeutic monitoring of organophosphate poisoned patients. Nevertheless, the procedure is still in an experimental stage, and no certified method is available. Major confounding factors are the high hemoglobin absorption, the changes of methemoglobin absorption in the presence of detergents (particularly pronounced in lyophilized samples), the thermochromic shifts of the indicator absorbance, the interference of butyrylcholinesterase, and the appropriate sample storage when an anticholinesterase and reactivator are present. Here, we present a modified approach that requires only low-tech laboratory equipment (filter photometer with mercury lamp) and gives reliable results down to 3% residual activity. To increase the signal-to-noise ratio, the color development is measured at 436 nm, the acetylthiocholine concentration is reduced to 0.45 mM, and the pH is lowered to 7.4. Hemolysis is achieved by Triton X-100, with its final concentration not exceeding 0.01%. Ery-AChE activity is referred to the hemoglobin content, determined as cyanmethomoglobin at 546 nm. Butyrylcholinesterase activity is inhibited by 20 μM ethopropazine. The within-run precision was 1 and 5% in native and inhibited (95%) samples. When diluted 1:100 and frozen at -20°C, paraoxon-inibited samples were stable over 1 month even in the presence of 10 μM obidoxime.

INTRODUCTION

The determination of acetylcholinesterase (AChE, EC 3.1.1.7) activity is important in
1)monitoring and studying of exposure to pesticides (1) and chemical warfare agents (2);
2) therapeutic monitoring of organophosphate poisoned patients (3); and
3) titrating the anticholinesterase dosage used in Alzheimer's disease (4).

While the most interesting muscular and neuronal AChE is not accessible to direct measurement, Ery-AChE can easily be obtained. This source is regarded to be a reliable surrogate marker (5), because of the structural and functional similarities due to the common genetic origin of the catalytic subunit (6).

A variety of methods (electrometry, pH-stat, tintometry, radiometry and colorimetry) have been developed, and each method has its distinct advocates (7; 8). The most widespread method is based on the hydrolysis of acetylthiocholine. The assay introduced by Ellman in 1961 (9) makes use of the thiocholine-mediated cleavage of the chromogenic disulfide DTNB. Although the method is rather rapid, simple, and cheap, the Ellman method has its limitations because the peak absorption around 412 nm of the colored indicator TNB^- coincides with the Soret band of mammalian hemoglobins. Moreover, various side reactions may falsify the determination of Ery-AChE activity. For these reasons we felt it necessary to modify the original Ellman method and to develop a robust procedure for estimating AChE activity in whole blood samples.

The method was optimized for human blood to also allow the detection of small activities of Ery-AChE in intoxicated patients. Hence, major efforts have been undertaken to reduce annoying side-effects and to optimize the signal-to-noise ratio. To this end, we lowered the reaction pH to 7.4 instead of 8.0 and reduced the substrate concentration from 1.0 to 0.45 mM, i.e. 5 x K_m, resulting in 83% V_{max}. Thereby, the blank reaction was reduced to about 1% of normal Ery-AChE activity, which allows a detection limit of 3% of normal (10).

Selection of a suitable wavelength for the Ellman assay

The interference of hemoglobin absorption can be circumvented by measuring TNB^- outside its absorbance maximum. We selected 436 nm wavelength, since this wavelength is available in simple filter photometers equipped with mercury lamps. Thereby exact reproducibility of wavelength and band width are guaranteed without the need of a sophisticated spectrophotometer that would be a prerequisite if absorbance measurements are undertaken at a steep flank. At 436 nm the hemoglobin absorption is reduced to one fourth compared to 412 nm, while the indicator absorption is still 80% of its maximum. Thus, the signal-to-noise ratio is increased by a factor of three (10).

Minimizing of side reactions

Background changes of the sample matrix are of major concern. Thiol groups in the blood sample, most of all in glutathione, hemoglobin, and albumin, react with DTNB at widely varying rates, also differing among species. Because these reactions are complete in 5-6 min in human blood samples at 0.3 mM DTNB, pH 7.4 and 37°C, we have adopted a blank reaction period of 10 min (10). After this time also the temperature of the cuvette is

equilibrated to 37°C, which improves the assay reproducibility. A reaction temperature of 37°C was chosen, because this temperature is generally recommended when determining human enzymes. Moreover, adjustment to this temperature usually does not require an additional cooling device (opposed to 25°C reaction temperature). This aspect may be most important in countries with hot climate.

If the sample contains methemoglobin, particularly high in frozen samples or in lyophilized specimens that may be used as standards, slow absorption change of this pigment is observed when detergents are present in order to facilitate hemolysis (10). Hence we keep the Triton X-100 concentration in the assay very low (final concentration 0.01%).

Last but not least, butyrylcholinesterase (BChE, EC 3.1.1.8), particularly in human plasma, markedly contributes to acetylthiocholine hydrolysis. Using packed or washed red cells, or inclusion of "selective" BChE inhibitors, are the most common means to tackle this problem (2). Since we are working with frozen and hence hemolysed samples, procedures to physically remove BChE are inappropriate. Hence, we include ethopropazine as a quite selective BChE inhibitor (11). At a final concentration of 20 µM, BChE is inhibited by 97% while AChE is reduced by approx. 5% (10). We regard this small, but reproducible effect insignificant in view of the advantages to preserve the activity in frozen samples.

Sample storage

Inadequate sample storage is another factor that may confound AChE determination. While the high stability of native AChE usually does not pose major problems, the presence of anticholinesterases and/or reactivators does. For example, paraoxon-inhibited Ery-AChE shows a reactivation *ex vivo* with a half-time of some min at therapeutic oxime concentrations (12). To obtain reliable results, such a reaction has to be stopped immediately after blood sampling.

In order to minimize reactions between AChE, inhibitor and reactivator, immediate dilution of whole blood samples after withdrawal was considered a convenient method, feasible also in a busy emergency room. An approx. 1:100 dilution of whole blood in the assay buffer slows down bimolecular reactions by a factor of 10.000. The samples can be kept in a freezer at -20°C, allowing transportation, storage, and analysis up to 1 month later without significant changes. To correct for dilution errors, the enzyme activity is referred to the hemoglobin content (13; 14), measured as cyanomethemoglobin by a modified Zijlstra method (15) at 546 nm (another mercury emission line). Care is to be taken to avoid cyanide loss in the transformation solution which can be minimized (10) by the addition of sodium bicarbonate, pH 8.8, instead of the primary phosphate as used in the original Zijlstra method (pH 7.3). All these considerations have led to the following SOPs (16).

MATERIALS AND METHODS

SOP Sample Handling

Mix 0.2 ml (preferably taken with an insulin syringe) of venous blood (EDTA or heparin) immediately with 20 ml diluting reagent and store in a freezer at -20°C until shipment (dry ice) or analysis. Ideally, the diluting reagent is composed of 0.1 M sodium phosphate buffer, pH 7.4 containing 0.03% Triton X-100 to facilitate complete hemolysis. We use buffer-containing vials prepared in advance (Super polyethylene vials® for liquid scintillation counting, Packard) that are stored in the refrigerator. If need be, saline can be used instead as diluting reagent. The samples are thawed by gentle shaking the vials in cold water (conveniently in a gyrotory water bath shaker) and kept on ice until analysis.

SOP Determination of AChE Activity

Mix in polystyrene cuvettes:	final conc. (mM)
2.000 mL sodium phosphate buffer (0.1 M; pH 7.4)	100
0.100 mL DTNB (10 mM)	0.3
0.010 mL ethopropazine (6 mM)	0.02
1.000 mL hemolysate (whole blood 1:100)	
Equilibrate at 37°C for 10 min, then add:	
0.050 mL acetylthiocholine (28.4 mM)	0.45
Record color development for 3 min at 436 nm	
(e.g. in a filter photometer at 1.0 AUFS; _ = $10.6 \times 10^3 M^{-1} cm^{-1}$).	
Correct for spontaneous substrate hydrolysis (substitute hemolysate by buffer).	
Typical readings: Hemolysate	170 mE/min
Blank	2 mE/min

SOP Total hemoglobin

Mix 1.00 mL hemolysate (whole blood 1:100) with 1.00 mL modified Zijlstra reagent and incubate for 10 min at room temperature.

Read extinction at 546 nm (room temperature or 37°C)

(e.g. in a filter photometer at 1.0 AUFS; _ = $10.8 \times 10^3 M^{-1} cm^{-1}$).
Typical reading: 0.48 E; corresponding to 44 µM hemoglobin iron.
For calculation of the specific activity of Ery-AChE, presented as U/µmol Hb(Fe), calculate the quotient of AChE activity (µM/min) and µM Hb as obtained from the measurements in hemolysate (whole blood 1:100) and multiply with 1.58 to correct for different dilutions in determining hemoglobin and AChE.
Typical activity of normal Ery-AChE: 0.6 U/µmol Hb(Fe).
By multiplying this value with 62 one gets the dimension U/g hemoglobin.

RESULTS

Assay linearity

The linearity of the assay response to different enzyme activities was tested in the presence of the same matrix concentration. This was achieved by mixing inhibited AChE with various proportions of active enzyme. As shown in Fig. 1 the correlation was linear and intersected the y-axis at a blank rate of roughly 2 mE/min. A linear correlation was found between AChE activity and hemoglobin content when the same sample of whole blood was diluted at 1:50 to 1:200 (Fig. 2). Hence, exact dilution of freshly withdrawn blood is not critical. The specific activity in these samples averaged 0.623 U/µmol Hb at a CV of 1.26%.

Within-run precision

Repetitive measurements of native samples gave excellent reproducibility (Table 1). A comparable within-run precision was obtained with blood samples in the presence of paraoxon-ethyl, paraoxon-methyl and obidoxime at its therapeutic concentration of 10 µM (3), resulting in inhibition between 10 and 94% of control.

Between-run precision

The determination of AChE activities in a native sample over 5 consecutive days resulted in acceptable reproducibility with a CV of 3% (Table 2). Repetitive freezing and thawing of whole blood dilutions over 3 days had no effect on AChE activity (CV 1.35%) or hemoglobin content (CV 0.70%).

AChE stability

AChE activity in diluted, frozen whole blood samples was stable for at least 7 days and decreased slowly to 91.7 ± 1.8% within 5 weeks. The inhibition brought about by paraoxon-ethyl or paraoxon-methyl remained constant for a month even in the presence of free inhibitor and obidoxime (Fig. 3).

CONCLUSIONS

The data presented show that reliable measurements of Ery-AChE activity can be easily performed with low-tech equipment and under conditions that are likely to be found at the scene where intoxications are to be expected. The sample handling is easy, exact dilution is not necessary, the only prerequisite is an effective cooling facility (freezer). Enzymatic analysis does not need a centrifuge but only a simple thermostated filter photometer to determine enzyme activity and to allow reference to the hemoglobin content. The described procedure is intended for manual use, but should be easily adapted to autoanalyzers or microtiter plate procedures if large quantities of samples are to be measured.

It should be noted that determination of plasma cholinesterase should be possible in the diluted whole blood samples on substituting acetylthiocholine by butyrylthiocholine and omission of the BChE inhibitor. Alternatively, BChE activity can be easily determined in serum or plasma, which, however, requires rapid physical separation from the blood cells (10).

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KEYWORDS
Ellman assay, acetylcholinesterase, organophosphates, erythrocytes

FIGURES AND TABLES

Figure. 1 Assay linearity of different enzyme activities at constant sample matrix.
Inhibited AChE samples were mixed with various proportions of native enzyme (n=2). The data are shown as mE/min (mean ± SD; $r^2 = 0.9975$).

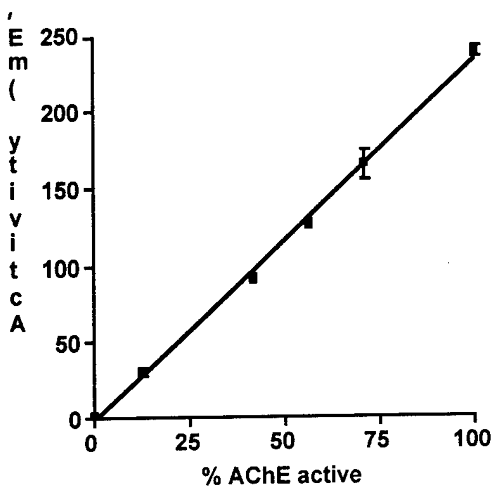

Figure. 2 Correlation of AChE activity and hemoglobin content in differently diluted whole blood samples (1:50 to 1:200, n=2, mean ± SD; $r^2 = 0.9996$).

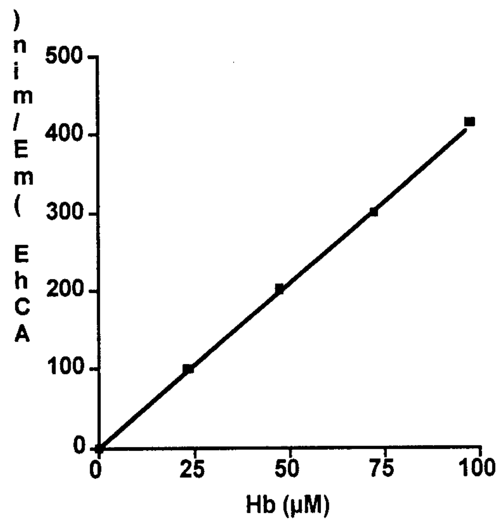
AChE (mE/min)
500
400
300
200
100
0
0
25
50
75
100
Hb (μM)

Figure. 3 Persistence of AChE inhibition during storage at -20°C.
Whole blood was inhibited with paraoxon-methyl (3 μM) or paraoxon-ethyl (1 μM) in the presence of obidoxime (10 μM) for 15 min at 37°C, followed by immediate dilution of the blood samples (1:100). Samples (n=5) were thawed after different periods and analyzed in the modified Ellman assay (mean ± SD).

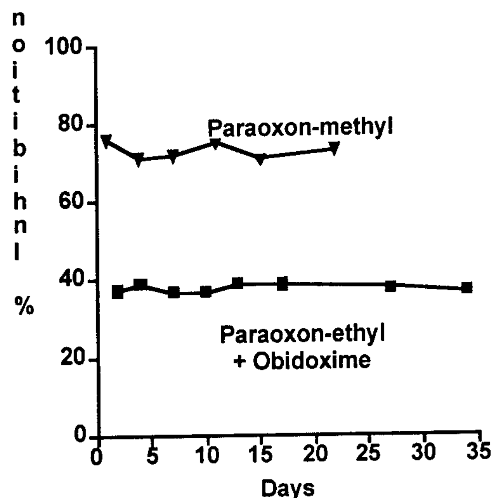

Table 1 Within-run precision of Ery-AChE determination in whole blood

Paraoxon-ethyl (μM)	Obidoxime (μM)	Activity (mU/μmol Hb)	CV (%)	Inhibition (%)
0	0	586±5	0.95	0
0.2	0	32±1	4.26	94
0.2	10	524±2	0.50	11
1.0	10	224±2	1.05	64

Blood was incubated with inhibitor and oxime for 15 min at 37°C, as indicated (n=10).
Immediately thereafter blood was diluted (1:100) and all samples were frozen and analyzed the next day.
The specific activity is given as mean ± SD and the assay reproducibility as coefficient of variation (CV).

Table 2. Between-run precision of Ery-AChE determination in whole blood

Activity (mU/μmol Hb)	CV (%)
651±18	0

Whole blood dilutions (n=25) were assayed on five consecutive days. The specific activity is given as means ± SD and the assay reproducibility as coefficient of variation (CV).

30. THE DETERMINATION OF HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE (ERY-ACHE) IN A MODIFIED ELLMAN ASSAY

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ABSTRACT

The colorimetric Ellman method is the most commonly used assay for the determination of human Ery-AChE in occupational health screening and therapeutic monitoring of organophosphate poisoned patients. Nevertheless, the procedure is still in an experimental stage, and no certified method is available. Major confounding factors are the high hemoglobin absorption, the changes of methemoglobin absorption in the presence of detergents (particularly pronounced in lyophilized samples), the thermochromic shifts of the indicator absorbance, the interference of butyrylcholinesterase, and the appropriate sample storage when an anticholinesterase and reactivator are present. Here, we present a modified approach that requires only low-tech laboratory equipment (filter photometer with mercury lamp) and gives reliable results down to 3% residual activity. To increase the signal-to-noise ratio, the color development is measured at 436 nm, the acetylthiocholine concentration is reduced to 0.45 mM, and the pH is lowered to 7.4. Hemolysis is achieved by Triton X-100, with its final concentration not exceeding 0.01%. Ery-AChE activity is referred to the hemoglobin content, determined as cyanmethomoglobin at 546 nm. Butyrylcholinesterase activity is inhibited by 20 µM ethopropazine. The within-run precision was 1 and 5% in native and inhibited (95%) samples. When diluted 1:100 and frozen at -20°C, paraoxon-inibited samples were stable over 1 month even in the presence of 10 µM obidoxime.

INTRODUCTION

The determination of acetylcholinesterase (AChE, EC 3.1.1.7) activity is important in
1)monitoring and studying of exposure to pesticides (1) and chemical warfare agents (2);
2) therapeutic monitoring of organophosphate poisoned patients (3); and
3) titrating the anticholinesterase dosage used in Alzheimer's disease (4).

While the most interesting muscular and neuronal AChE is not accessible to direct measurement, Ery-AChE can easily be obtained. This source is regarded to be a reliable surrogate marker (5), because of the structural and functional similarities due to the common genetic origin of the catalytic subunit (6).

A variety of methods (electrometry, pH-stat, tintometry, radiometry and colorimetry) have been developed, and each method has its distinct advocates (7; 8). The most widespread method is based on the hydrolysis of acetylthiocholine. The assay introduced by Ellman in 1961 (9) makes use of the thiocholine-mediated cleavage of the chromogenic disulfide DTNB. Although the method is rather rapid, simple, and cheap, the Ellman method has its limitations because the peak absorption around 412 nm of the colored indicator TNB^- coincides with the Soret band of mammalian hemoglobins. Moreover, various side reactions may falsify the determination of Ery-AChE activity. For these reasons we felt it necessary to modify the original Ellman method and to develop a robust procedure for estimating AChE activity in whole blood samples.

The method was optimized for human blood to also allow the detection of small activities of Ery-AChE in intoxicated patients. Hence, major efforts have been undertaken to reduce annoying side-effects and to optimize the signal-to-noise ratio. To this end, we lowered the reaction pH to 7.4 instead of 8.0 and reduced the substrate concentration from 1.0 to 0.45 mM, i.e. 5 x K_m, resulting in 83% V_{max}. Thereby, the blank reaction was reduced to about 1% of normal Ery-AChE activity, which allows a detection limit of 3% of normal (10).

Selection of a suitable wavelength for the Ellman assay

The interference of hemoglobin absorption can be circumvented by measuring TNB^- outside its absorbance maximum. We selected 436 nm wavelength, since this wavelength is available in simple filter photometers equipped with mercury lamps. Thereby exact reproducibility of wavelength and band width are guaranteed without the need of a sophisticated spectrophotometer that would be a prerequisite if absorbance measurements are undertaken at a steep flank. At 436 nm the hemoglobin absorption is reduced to one fourth compared to 412 nm, while the indicator absorption is still 80% of its maximum. Thus, the signal-to-noise ratio is increased by a factor of three (10).

Minimizing of side reactions

Background changes of the sample matrix are of major concern. Thiol groups in the blood sample, most of all in glutathione, hemoglobin, and albumin, react with DTNB at widely varying rates, also differing among species. Because these reactions are complete in 5-6 min in human blood samples at 0.3 mM DTNB, pH 7.4 and 37°C, we have adopted a blank reaction period of 10 min (10). After this time also the temperature of the cuvette is

equilibrated to 37°C, which improves the assay reproducibility. A reaction temperature of 37°C was chosen, because this temperature is generally recommended when determining human enzymes. Moreover, adjustment to this temperature usually does not require an additional cooling device (opposed to 25°C reaction temperature). This aspect may be most important in countries with hot climate.

If the sample contains methemoglobin, particularly high in frozen samples or in lyophilized specimens that may be used as standards, slow absorption change of this pigment is observed when detergents are present in order to facilitate hemolysis (10). Hence we keep the Triton X-100 concentration in the assay very low (final concentration 0.01%).

Last but not least, butyrylcholinesterase (BChE, EC 3.1.1.8), particularly in human plasma, markedly contributes to acetylthiocholine hydrolysis. Using packed or washed red cells, or inclusion of "selective" BChE inhibitors, are the most common means to tackle this problem (2). Since we are working with frozen and hence hemolysed samples, procedures to physically remove BChE are inappropriate. Hence, we include ethopropazine as a quite selective BChE inhibitor (11). At a final concentration of 20 µM, BChE is inhibited by 97% while AChE is reduced by approx. 5% (10). We regard this small, but reproducible effect insignificant in view of the advantages to preserve the activity in frozen samples.

Sample storage

Inadequate sample storage is another factor that may confound AChE determination. While the high stability of native AChE usually does not pose major problems, the presence of anticholinesterases and/or reactivators does. For example, paraoxon-inhibited Ery-AChE shows a reactivation *ex vivo* with a half-time of some min at therapeutic oxime concentrations (12). To obtain reliable results, such a reaction has to be stopped immediately after blood sampling.

In order to minimize reactions between AChE, inhibitor and reactivator, immediate dilution of whole blood samples after withdrawal was considered a convenient method, feasible also in a busy emergency room. An approx. 1:100 dilution of whole blood in the assay buffer slows down bimolecular reactions by a factor of 10.000. The samples can be kept in a freezer at -20°C, allowing transportation, storage, and analysis up to 1 month later without significant changes. To correct for dilution errors, the enzyme activity is referred to the hemoglobin content (13; 14), measured as cyanomethemoglobin by a modified Zijlstra method (15) at 546 nm (another mercury emission line). Care is to be taken to avoid cyanide loss in the transformation solution which can be minimized (10) by the addition of sodium bicarbonate, pH 8.8, instead of the primary phosphate as used in the original Zijlstra method (pH 7.3). All these considerations have led to the following SOPs (16).

MATERIALS AND METHODS

SOP Sample Handling

Mix 0.2 ml (preferably taken with an insulin syringe) of venous blood (EDTA or heparin) immediately with 20 ml diluting reagent and store in a freezer at -20°C until shipment (dry ice) or analysis. Ideally, the diluting reagent is composed of 0.1 M sodium phosphate buffer, pH 7.4 containing 0.03% Triton X-100 to facilitate complete hemolysis. We use buffer-containing vials prepared in advance (Super polyethylene vials® for liquid scintillation counting, Packard) that are stored in the refrigerator. If need be, saline can be used instead as diluting reagent. The samples are thawed by gentle shaking the vials in cold water (conveniently in a gyrotory water bath shaker) and kept on ice until analysis.

SOP Determination of AChE Activity

Mix in polystyrene cuvettes:	final conc. (mM)
2.000 mL sodium phosphate buffer (0.1 M; pH 7.4)	100
0.100 mL DTNB (10 mM)	0.3
0.010 mL ethopropazine (6 mM)	0.02
1.000 mL hemolysate (whole blood 1:100)	
Equilibrate at 37°C for 10 min, then add:	
0.050 mL acetylthiocholine (28.4 mM)	0.45

Record color development for 3 min at 436 nm

(e.g. in a filter photometer at 1.0 AUFS; _ = $10.6 \times 10^3 M^{-1} cm^{-1}$).

Correct for spontaneous substrate hydrolysis (substitute hemolysate by buffer).

Typical readings: Hemolysate	170 mE/min
Blank	2 mE/min

SOP Total hemoglobin

Mix 1.00 mL hemolysate (whole blood 1:100) with 1.00 mL modified Zijlstra reagent and incubate for 10 min at room temperature.

Read extinction at 546 nm (room temperature or 37°C)

(e.g. in a filter photometer at 1.0 AUFS; _ = 10.8 x 10^3 M^{-1} cm^{-1}).
Typical reading: 0.48 E; corresponding to 44 μM hemoglobin iron.
For calculation of the specific activity of Ery-AChE, presented as U/μmol Hb(Fe), calculate the quotient of AChE activity (μM/min) and μM Hb as obtained from the measurements in hemolysate (whole blood 1:100) and multiply with 1.58 to correct for different dilutions in determining hemoglobin and AChE.
Typical activity of normal Ery-AChE: 0.6 U/μmol Hb(Fe).
By multiplying this value with 62 one gets the dimension U/g hemoglobin.

RESULTS

Assay linearity

The linearity of the assay response to different enzyme activities was tested in the presence of the same matrix concentration. This was achieved by mixing inhibited AChE with various proportions of active enzyme. As shown in Fig. 1 the correlation was linear and intersected the y-axis at a blank rate of roughly 2 mE/min. A linear correlation was found between AChE activity and hemoglobin content when the same sample of whole blood was diluted at 1:50 to 1:200 (Fig. 2). Hence, exact dilution of freshly withdrawn blood is not critical. The specific activity in these samples averaged 0.623 U/μmol Hb at a CV of 1.26%.

Within-run precision

Repetitive measurements of native samples gave excellent reproducibility (Table 1). A comparable within-run precision was obtained with blood samples in the presence of paraoxon-ethyl, paraoxon-methyl and obidoxime at its therapeutic concentration of 10 μM (3), resulting in inhibition between 10 and 94% of control.

Between-run precision

The determination of AChE activities in a native sample over 5 consecutive days resulted in acceptable reproducibility with a CV of 3% (Table 2). Repetitive freezing and thawing of whole blood dilutions over 3 days had no effect on AChE activity (CV 1.35%) or hemoglobin content (CV 0.70%).

AChE stability

AChE activity in diluted, frozen whole blood samples was stable for at least 7 days and decreased slowly to 91.7 ± 1.8% within 5 weeks. The inhibition brought about by paraoxon-ethyl or paraoxon-methyl remained constant for a month even in the presence of free inhibitor and obidoxime (Fig. 3).

CONCLUSIONS

The data presented show that reliable measurements of Ery-AChE activity can be easily performed with low-tech equipment and under conditions that are likely to be found at the scene where intoxications are to be expected. The sample handling is easy, exact dilution is not necessary, the only prerequisite is an effective cooling facility (freezer). Enzymatic analysis does not need a centrifuge but only a simple thermostated filter photometer to determine enzyme activity and to allow reference to the hemoglobin content. The described procedure is intended for manual use, but should be easily adapted to autoanalyzers or microtiter plate procedures if large quantities of samples are to be measured.

It should be noted that determination of plasma cholinesterase should be possible in the diluted whole blood samples on substituting acetylthiocholine by butyrylthiocholine and omission of the BChE inhibitor. Alternatively, BChE activity can be easily determined in serum or plasma, which, however, requires rapid physical separation from the blood cells (10).

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KEYWORDS
Ellman assay, acetylcholinesterase, organophosphates, erythrocytes

FIGURES AND TABLES

Figure. 1 Assay linearity of different enzyme activities at constant sample matrix.
Inhibited AChE samples were mixed with various proportions of native enzyme (n=2). The data are shown as mE/min (mean ± SD; $r^2 = 0.9975$).

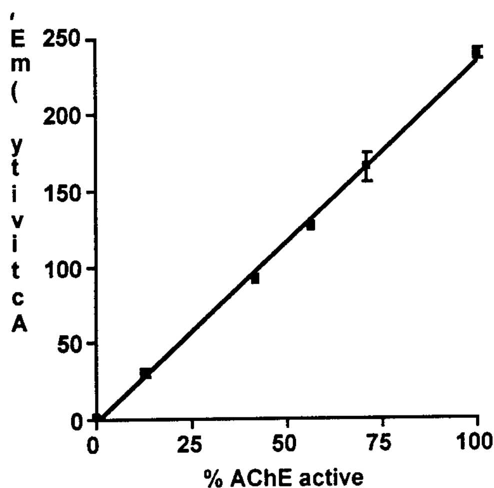

Figure. 2 Correlation of AChE activity and hemoglobin content in differently diluted whole blood samples (1:50 to 1:200, n=2, mean ± SD; $r^2 = 0.9996$).

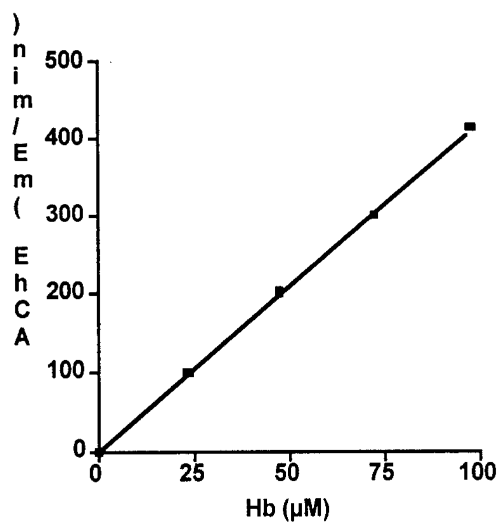
AChE (mE/min)
500
400
300
200
100
0
0
25
50
75
100
Hb (μM)

Figure. 3 Persistence of AChE inhibition during storage at -20°C.
Whole blood was inhibited with paraoxon-methyl (3 µM) or paraoxon-ethyl (1 µM) in the presence of obidoxime (10 µM) for 15 min at 37°C, followed by immediate dilution of the blood samples (1:100). Samples (n=5) were thawed after different periods and analyzed in the modified Ellman assay (mean ± SD).

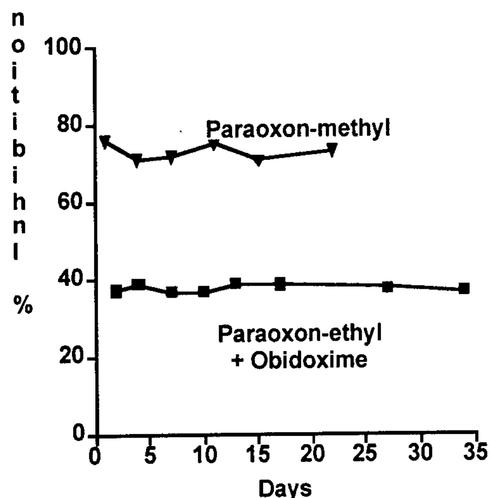

Table 1 Within-run precision of Ery-AChE determination in whole blood

Paraoxon-ethyl (µM)	Obidoxime (µM)	Activity (mU/µmol Hb)	CV (%)	Inhibition (%)
0	0	586±5	0.95	0
0.2	0	32±1	4.26	94
0.2	10	524±2	0.50	11
1.0	10	224±2	1.05	64

Blood was incubated with inhibitor and oxime for 15 min at 37°C, as indicated (n=10).
Immediately thereafter blood was diluted (1:100) and all samples were frozen and analyzed the next day.
The specific activity is given as mean ± SD and the assay reproducibility as coefficient of variation (CV).

Table 2. Between-run precision of Ery-AChE determination in whole blood

Activity (mU/µmol Hb)	CV (%)
651±18	0

Whole blood dilutions (n=25) were assayed on five consecutive days. The specific activity is given as means ± SD and the assay reproducibility as coefficient of variation (CV).

31. SULFUR MUSTARD INDUCED CYTOKINE CHANGES IN DIFFERENT CELL CULTURES

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ABSTRACT

Sulfur mustard (SM) is a vesicant, which is still a threat in war and terroristic attacks. The clinical effects of SM are delayed. Erythema, blisters and necrosis of the skin appear several hours after exposure. Although the role of inflammatory cytokines released after chemical injury is well known, the respective effects of SM are not well investigated. Especially interesting appears the symptom-free period with the beginning of the inflammatory process including cytokine changes.

Therefore, the release of cytokines in the first eight hours after SM exposure was investigated in human keratinocytes and in two permanent, related cell lines (SCL II, HaCaT). The keratinocytes were cultured in serum free KGM-2. SCL II cells were cultured in DMEM/F12 1:1 supplemented with glutamine and 10% FCS, the HaCaT cells in DMEM with 10% FCS. After reaching about 70% confluence the medium was replaced by a serum-free medium containing SM (500 µM) and exposed for 30 minutes. After exposure the medium was changed to the original one. Hourly samples of the supernatant were taken and the cytokine concentrations were measured with ELISA.

All investigated cell types showed an increased IL-6 and IL-8 secretion after SM exposure, whereas IL-1_ and IL-10 remained unchanged.

Increased TNF-_ and IL-1_ concentrations were found in HaCaT cell and keratinocyte supernatants, whereas no increase could be observed in SCL II cultures.

The data indicate HaCaT cells to be a more appropriate model for the study of SM effects in keratinocytes compared to SCL II cells.

INTRODUCTION

Sulfur mustard [Bis-(2-chlorethyl) sulfide, SM] is a strong alkylating agent. Its ability to induce DNA damage is well established (Szinicz and Baskin 1999). Skin exposure to sulfur mustard results in erythema and blistering via poorly understood mechanisms. The pathology of sulfur mustard injury includes an acute inflammatory response, serum leakage, microblister formation and signs of apoptosis and necrosis (Smith et al., 1995). 95% of the human epidermal cells are keratinocytes, which participate actively in epidermal response against external injury via cytokine release (Barker et al., 1991). Only limited data exist about the response of keratinocytes to sulfur mustard. This study describes the early responses of a human squamous cell carcinoma line (SCL II), an immortalized keratinocyte cell line (HaCaT) and of normal human epidermal keratinocytes to sulfur mustard, defined by TNF-α, IL-1α, IL-1ß, IL-6, IL-8 and IL-10 release.

MATERIALS AND METHODS

A human keratinocyte cell line derived from a squamous cell carcinoma (SCL II) was cultured in Dulbecco's Modified Eagle Medium/Ham's F12 (Gibco, Eggenstein, FRG) with 2,45 mmol/L glutamine, 10% fetal calf serum (FCS, Boehringer, Mannheim, FRG), 50 U/mL Penicillin and 50 µg/mL Streptomycin (Gibco, Eggenstein, FRG) in 75 cm_ flasks (Falcon, Heidelberg, FRG) in humidified atmosphere with 5% CO_2 at 37°C (Tilgen et al. 1983). HaCaT cells, were cultured like SCL II cells in Dulbecco's Modified Eagle Medium supplemented with 10% FCS as described by Breitkreutz et al. 1998. SCL II and HaCaT cells had been drawn from a stock given to the Institute of Radiobiology, German Armed Forces Medical Academy, Munich, FRG, by Prof. Tilgen respectively Prof. Dr. W. Fusenig. The normal human epidermal keratinocytes (NHEK) were purchased from CellSystems, St. Katharinen, FRG, or were isolated by us from foreskin (Saffran et al. 1997). The keratinocytes were cultured in KGM-2 (keratinocyte growth medium-2; CellSystems). The optimized medium was supplemented with 0.1 ng/mL human recombinant Epidermal Growth Factor, 5 µg/mL Insulin, 0.5 µg/mL Hydrocortisone, 50 µg/mL Gentamicin, 50 ng/mL Amphotericin B and 15 mg bovine pitutary extract. The keratinocytes were also cultured at 37°C in a humidified atmosphere with 5% CO_2.

Cytokine release was measured with ELISA (Coulter Immunotech, FRG) according to the manufacturer's instructions.

RESULTS

All cell lines showed a significant increase in IL-6 and IL-8 release during 8 h after sulfur mustard exposure (two way ANOVA $p \leq 0.05$). IL-1ß and IL-10 levels remained unchanged. Sulfur mustard induced a significant release of TNF-α and IL-1α only in HaCaT cell cultures and human keratinocytes (table 1).

CONCLUSIONS

Although "primed to die", the investigated cell lines were able to release several cytokines in the "early phase" after sulfur mustard exposure. This possibly initiates the inflammatory response to sulfur mustard. TNF-α and IL-1 release result in an activation of dermal endothelial cells. IL-8 promotes T cell migration toward epidermis. Compared to literature HaCaT cells showed the same cytokine release profile as keratinocytes activated by UVB irradiation (Barker et al. 1991). Interestingly, no increase of IL-1α and TNF-α formation was observed in SCL II cell cultures. The data demonstrate relevant cytokine changes short after sulfur mustard exposure, which might represent an early step in induction of cell death by the toxicant.

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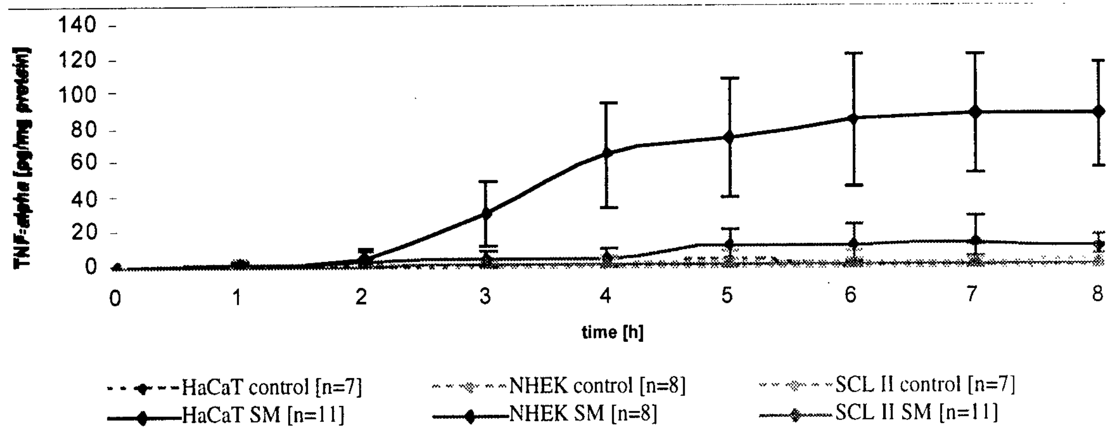
KEYWORDS

Cytokine-HaCaT-Keratinocyte-SCL II- Sulfur mustard

FIGURES AND TABLES

Figure 1. SCL II, HaCaT cells, and human keratinocytes were exposed to 500 μM sulfur mustard for 30 minutes. TNF-α was measured in the supernatant at various times after exposure.



140
120
100
80
60
40
20
0
TNF-alpha [pg/mg protein]
0
1
2
3
4
5
6
7
8
time [h]
HaCaT control [n=7]
NHEK control [n=8]
SCL II control [n=7]
HaCaT SM [n=11]
NHEK SM [n=8]
SCL II SM [n=11]

Figure 2. SCL II, HaCaT cells, and human keratinocytes were exposed to 500 µM sulfur mustard for 30 minutes. IL-6 was measured in the supernatant at various times after exposure.

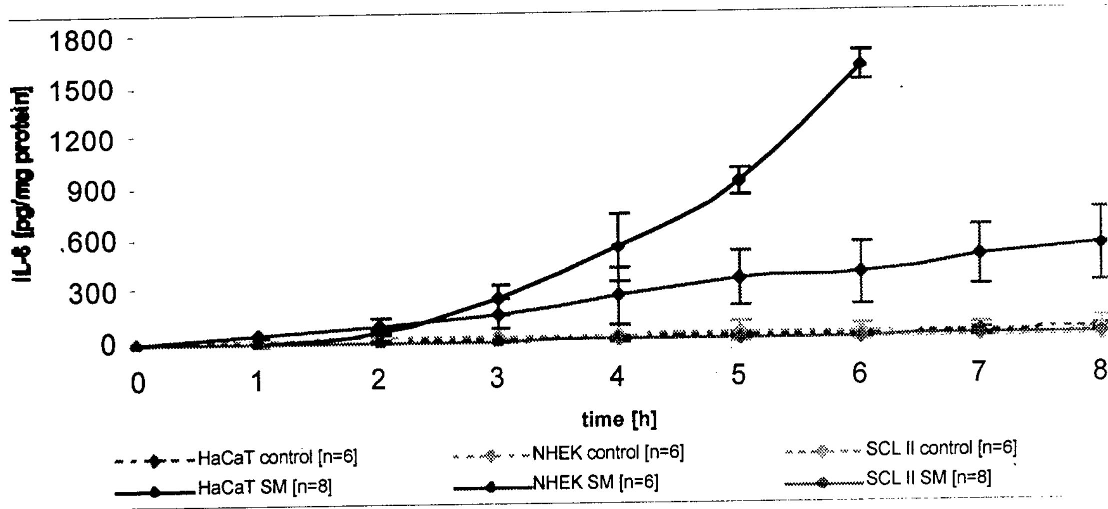


Figure 3. SCL II, HaCaT cells, and human keratinocytes were exposed to 500 µM sulfur mustard for 30 minutes. IL-8 was measured in the supernatant at various times after exposure.

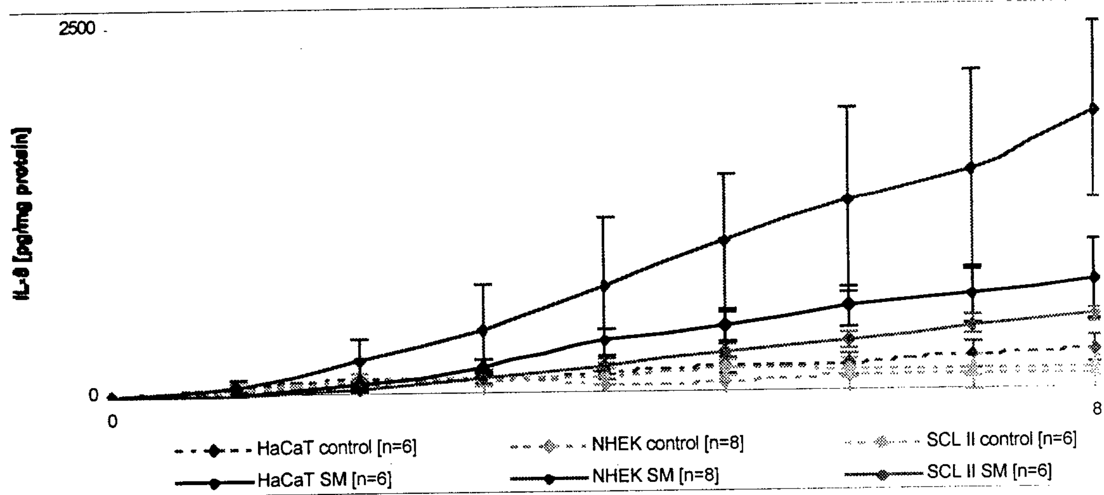


Table 1. Effects of SM on the release of various cytokines in supernatants of different cell cultures. Cytokine concentration expressed in pg/mg protein, measured 8 hours after exposure.
sham treated = nutrient medium treated = 500 μM sulfur mustard for 30 minutes
nd = not detectable

	SCL II		HaCaT		NHEK	
cytokine	sham treated	treated	sham treated	treated	sham treated	treated
IL-1a	1	nd	1	61	15	49
IL-1ß	nd	nd	nd	nd	nd	nd
IL-6	12	34	56	> 1600	41	541
IL-8	140	490	262	733	110	1891
IL-10	nd	nd	nd	nd	nd	nd
TNF-a	0.4	1.5	4	88	4	12

32. NEW ASPECTS ON THE REACTIVATION BY OXIMES OF ORGANOPHOSPHATE-INHIBITED HUMAN ACETYLCHOLINESTERASE IN VITRO

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INTRODUCTION

Recent incidents remind that highly toxic organophosphonates (nerve agents), e.g. sarin, still have to be considered a real threat for the population (1). In contrast to organophosphate insecticides, intoxications with organophosphonates result in phosphonylated acetylcholinesterase (AChE) which is notoriously resistant to reactivation by the marketed oximes, i.e. various pralidoxime salts and obidoxime (2, 3, 4). This fact prompted the synthesis of hundreds of oximes with numerous structural modifications. Recently, we determined the reactivating potency of the marketed oximes, obidoxime and pralidoxime (2-PAM), and of the potential successor oximes, HI 6 and HLö 7 (3, 4, 5, 6, 7). The available data suggest that obidoxime is the most effective reactivator of human AChE inhibited by pesticides resulting in diethylphosphoryl- (e.g. paraoxon) or dimethylphosphoryl-AChE (e.g. malaoxon). The bispyridinium-dioxime HLö 7 was slightly less effective whereas pralidoxime and HI 6 showed to be weak reactivators. According to these data obidoxime is considered to be the oxime of choice for the treatment of pesticide poisoning. In contrast, HLö 7 and HI 6 were superior to obidoxime and pralidoxime with sarin-, cyclosarin- and VX-inhibited AChE. Soman- and tabun-inhibited AChE were generally resistant to reactivation by oximes.

The resistance of phosphonylated AChE to reactivation by oximes may be attributed to rapid dealkylation (`aging´) or to a limited accessibility of the active site of AChE for the oximes.

A closer look at the reactivation scheme for the removal by oximes of the phosphonyl moiety from the enzyme directs to another factor which can also affect the reactivation process (8, 9):

$$[EP] + [OX] \leftrightarrow [E] + [POX] \qquad (1)$$

According to this scheme, incubation of phosphonylated AChE [EP] with an oxime [OX] results in the formation of equimolar amounts of free enzyme [E] and of phosphonyloximes [POX, denoting both phosphorylated and phosphonylated oxime]. Consecutive experiments demonstrated the high inhibitory potency of POX towards AChE (10, 11, 12, 13), being one or two orders of magnitude more potent than their parent organophosphorus compounds. Inspection of scheme 1 reveals that the forward reaction is usually of pseudo-first order ([OX] > [EP]) while the back reaction is close to second-order with nearly equimolar [E] and [POX], if [POX] is sufficiently stable and [E] stems essentially from the reactivation. It follows that the extent of re-inhibition should correlate with the concentration of the inhibited enzyme. In vitro experiments with phosphonylated (14, 15) and phosphorylated AChE (7) confirmed the dependence between AChE concentration and extent of reactivation by a certain oxime. Thus, the reactivation curves usually deviate from the pseudo-first order kinetics and net reactivation stops when re-inhibition by POX equals reactivation.

In order to elucidate the underlying mechanism we developed a model with physiologic enzyme concentrations and all components of human blood which allows simulations of the in vivo situation. In addition, we wanted to know whether human plasma also affects the reactivation by oximes of phosphonylated AChE since a recent study showed that human plasma is capable of degrading dimethyl- and diethyl-POX formed during the reactivation of human AChE with obidoxime (16).

MATERIALS AND METHODS

Acetylthiocholine iodide (ASCh), S-butyrylthiocholine iodide (BSCh), 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB), pralidoxime chloride (2-PAM) and ethopropazine were obtained from Sigma (Deisenhofen, Germany) and obidoxime dichloride (obidoxime) was purchased from Duphar (Amsterdam, Netherlands). HI 6 (1-[[[4-aminocarbonyl)-pyridinio]-methoxy]-methyl]-2-[(hydroxyimino)methyl] pyridinium dichloride monohydrate) was kindly provided by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada) and HLö 7 (1-[[[4-aminocarbonyl)-pyridinio]-methoxy]-methyl]-2,4-bis-[(hydroxyimino) methyl] pyridinium dimethanesulfonate) was a custom synthesis by J. Braxmeier (Chemisches Labor, Döpshofen, Germany). Sarin was made available by the Ministry of Defence (Bonn, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Sarin stock solutions in 2-propanol (0.1% v/v) were appropriately diluted in distilled water just before the experiment. Oximes (50 mM) were prepared in distilled water and diluted as required in distilled water or phosphate buffer (0.1 M, pH 7.4) at the day of the experiment. All solutions were kept on ice until the experiment.

Packed erythrocytes and plasma were incubated with sarin at 37°C for 15 min to achieve an inhibition by >95%. Inhibited and native erythrocytes were washed three times with 2 volumes of phosphate buffer (0.1 M, pH 7.4) to remove residual inhibitor. Inhibited and native plasma samples were dialysed overnight at 4°C against dialysing reagent (0.01 M phosphate buffer pH 7.4, 0.9% NaCl, 1 mM $CaCl_2$) to remove excess inhibitor and to adjust the pH at 7.4 while preserving the plasma paraoxonase activity (17). Thereafter, inhibited and control samples were mixed according to the protocol shown in Table 1.

For reactivation studies, 300 µl samples were incubated with 3 µl oxime (obidoxime, 2-PAM, HI 6 or HLö 7; 20 µM) and aliquots (10 µl) were used for measurement of the AChE activity (ethopropazine was added to the cuvets if plasma was present).

AChE and BChE activities were measured spectrophotometrically (Cary 3Bio, Varian, Darmstadt) with a modified Ellman assay (18). For the determination of AChE activity in the presence of plasma the specific BChE inhibitor ethopropazine (20 µM) was added.

All experiments were performed at 37°C and pH 7.4, and the activities were corrected for spontaneous and oxime-induced hydrolysis of the substrate. All concentrations refer to final concentrations.

RESULTS AND DISCUSSION

When sarin-inhibited human erythrocytes (iERY) were used, the reactivation proceeded monophasically with 20 µM HI 6 and HLö 7 and biphasically with 20 µM obidoxime and 2-PAM (Fig. 1A). This indicates that the POX generated from obidoxime and 2-PAM is sufficiently stable to impair the net reactivation. The addition of control plasma to iERY (iERY+cPL) enhanced the reactivation by obidoxime and 2-PAM (Fig. 1B). When a mixture of inhibited erythrocytes and inhibited plasma (iERY+iPL) was used, the reactivation was markedly retarded by obidoxime and 2-PAM but was hardly affected by HI 6 or HLö 7 (Fig. 1C).

Since the retardation may be caused by POX arising upon oxime-induced reactivation of inhibited BChE, the oximes were further tested for their ability to reactivate sarin-inhibited plasma BChE. Obidoxime and 2-PAM were found to be much more potent reactivators of sarin-inhibited BChE than HI 6 or HLö 7 (Fig. 2). Although the concentration of inhibited BChE was quite low (plasma diluted 1:100 in phosphate buffer, Fig. 2A), the reactivation was limited to some 60% with obidoxime and 2-PAM, possibly by accumulation of POX. To prove this the reactivation experiments were also performed with concentrated plasma (Fig 2B). The net reactivation was generally low and particularly reduced with obidoxime and 2-PAM.

To get more insight into these reactions, inhibited plasma without free sarin was incubated with control erythrocytes (cERY+iPL) in the presence of 20 µM obidoxime or HI 6 (Fig. 3). BChE activity increased at the expense of AChE when obidoxime was used, i.e. the generated inhibitor reacted preferably with AChE. When HI 6 was used, the increase of BChE activity was much slower and the AChE activity was hardly affected. This suggests that the hydrolysis of the isopropylmethylphosphonyl-HI 6 proceeded fast enough to prevent inhibition of AChE at these conditions.

Part of these experiments were also performed with cyclosarin and VX. The preliminary data suggest that stable POX is also formed during the reactivation of VX-inhibited AChE with obidoxime and 2-PAM. However, the impairment of net reactivation in the presence of inhibited plasma is minor when compared to sarin. With cyclosarin-inhibited cholinesterases almost no re-inhibition by POX was observed when obidoxime was used. 2-PAM showed to be an extremely weak reactivator of cyclosarin-inhibited human AChE (6), therefore, an impairment of reactivation by POX was hardly visible.

The present study confirms previous observations that POX´s formed during reactivation of phosphonylated AChE by certain oximes are of sufficient stability to impair net reactivation (11, 12, 13). Obviously, the degradation of POX´s proceeds much faster with bispyridinium oximes bearing the oxime function in position 2 (HI 6, HLö 7) than with 4-pyridiniumaldoximes, e.g. obidoxime. This study also indicates that the isopropylmethyl-POX of 2-PAM is not as unstable as may be anticipated from the position of the aldoxime group (10) and confirms data obtained with sarin- (and VX-) inhibited bovine erythrocyte AChE (15).

The negative impact of phosphonylated BChE on the net reactivation of erythrocyte AChE, however, is a new, unexpected finding. Phosphonylated BChE seems to contribute to POX formation significantly. Due to the higher concentration of active sites in human whole blood (approx. 50 nM) reactivated BChE provides much more POX molecules than erythrocyte AChE (approx. 10 nM). This was demonstrated in the cross-over experiment when

inhibited BChE and active erythrocyte AChE were incubated with obidoxime: 1/3 of BChE was reactivated while 2/3 of AChE was inhibited (Fig. 3). The results also suggest a substantially higher affinity of POX for AChE. Phosphonylated BChE is regarded to be less amenable to reactivation by oximes than erythrocyte AChE (14, 19). With diluted plasma, however, the reactivation of isopropylmethylphosphonyl-BChE was quite rapid with obidoxime and, most surprisingly, also with 2-PAM, while HLö 7 and HI 6 were rather weak reactivators. A `good' reactivation of isopropylmethylphosphonyl-BChE by an oxime is of disadvantage, because AChE is the target that is to be reactivated. In human poisoning with both AChE and BChE being inhibited, plasma BChE may be reactivated at the expense of erythrocyte AChE.

Recently, it was shown that human plasma is capable of degrading dimethyl- and diethyl-POX of obidoxime and TMB-4 (16). The POX hydrolysing activity of human plasma varied widely among different subjects and did not follow the activity pattern of human serum paraoxonase. Unfortunately, the present data suggest that human plasma has no hydrolysing activity for POX formed of sarin, cyclosarin or VX and obidoxime or 2-PAM.

SUMMARY

Our results shed new light on the dynamics of inhibition, reactivation and re-inhibition under conditions that simulate the situation in vivo better than experiments can do in which only AChE is used. Thus, a high reactivating potency of an oxime for inhibited BChE may disqualify it for antidotal use if stable POX is formed. Work is in progress to investigate this problem also with other nerve agents (cyclosarin, VX) and organophosphate insecticides.

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KEYWORDS

Organophosphate, acetylcholinesterase, reactivation, oximes, human, phosphonyloxime.

FIGURES AND TABLES

Table 1. Preparation of blood samples for reactivation studies

	AChE	BChE
iERY	Inhibited packed erythrocytes	phosphate buffer (0.1 M, pH 7.4)
iERY+cPL	Inhibited packed erythrocytes	control plasma
iERY+iPL	Inhibited packed erythrocytes	inhibited plasma
cERY+iPL	Control erythrocytes	inhibited plasma
iPL	-	inhibited plasma

Sarin-inhibited or control packed erythrocytes and plasma (pH adjusted to 7.4 by dialysis) or phosphate buffer were mixed in equal volumes. Corresponding control samples were prepared with active enzymes.

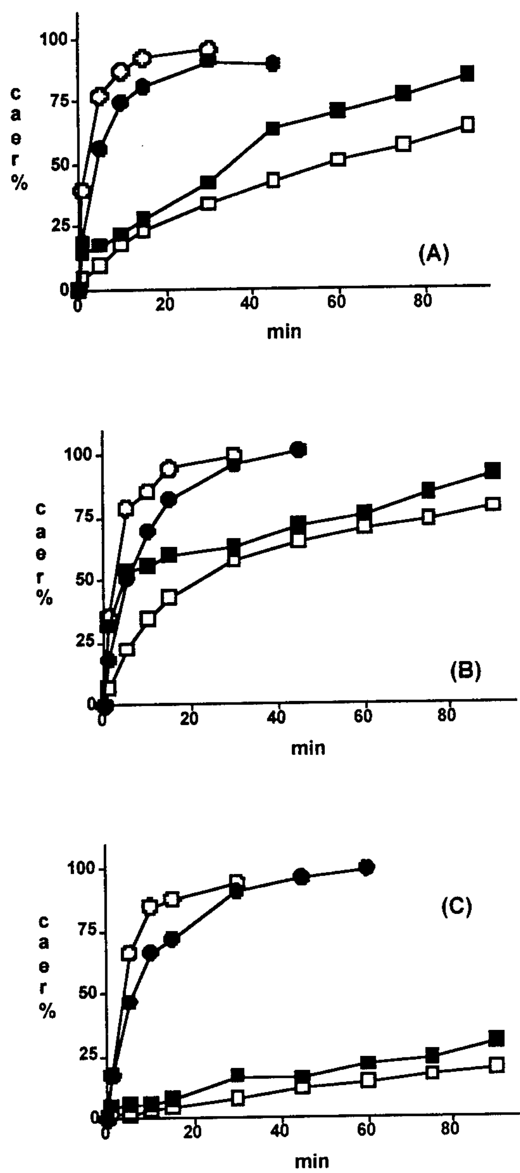
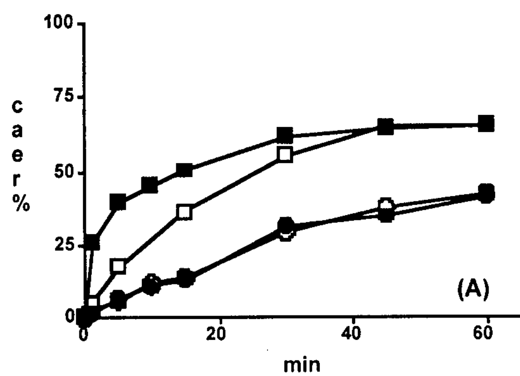


Figure 1. Reactivation of sarin-inhibited human AChE. Inhibited erythrocytes (iERY; A), inhibited erythrocytes plus control plasma (iERY+cPL; B), and inhibited erythrocytes plus inhibited plasma (iERY+iPL; C) were incubated with 20 µM obidoxime(■), 2-PAM (□), HI 6 (●), or HLö 7 (O). The data, expressed as %reac, are means of two experiments.



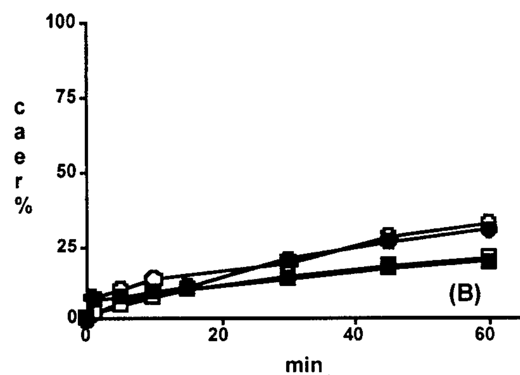
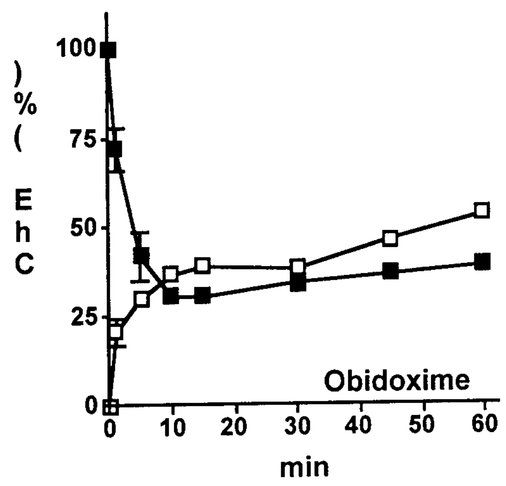


Figure 2. Reactivation of sarin-inhibited human BChE. Enzyme of diluted (1:100 in phosphate buffer 0.1 M, pH 7.4; A) and concentrated (B) sarin-inhibited plasma was reactivated with 20 μM obidoxime (■), 2-PAM (□), HI 6 (l), or HLö 7 (O). The data are given as %reac.



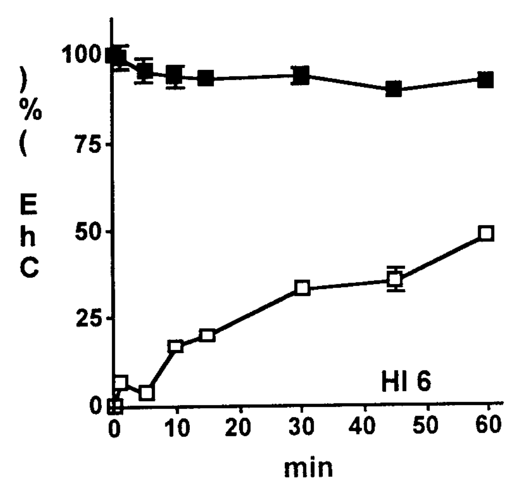


Figure 3. Effect of obidoxime (top) or HI 6 (bottom) on sarin-inhibited ChE. AChE (■) and BChE activities (□) were determined after the addition of obidoxime or HI 6 (20 μM) to a mixture of control erythrocytes and sarin-inhibited plasma (cERY+iPL).

33. PROPHYLACTIC EFFICACY OF AMIFOSTINE AND ITS ANALOGUES AGAINST SULPHUR MUSTARD INTOXICATION

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ABSTRACT

The successful ratification of the Chemical Weapons Convention (CWC) stimulated research on destruction of the stockpiled sulphur mustard (SM with a new vigor). During the destruction of the stockpiled SM and also during the inspections by the Organization for the Prohibition of Chemical Weapons (OPCW) a prophylactic agent for SM will be a useful requirement. Moreover, because the method of preparing SM is simple, the possibility of SM being used clandestinely during warfare or by terrorist groups still exists. Despite research over last several decades, no satisfactory prophylactic or treatment regimen has evolved for SM.

Amifostine, originally developed as a radioprotector, has been shown to be beneficial as a free radical scavenger in cancer chemotherapy. In animals, it protects normal tissues from the damaging effects of irradiation and several cytotoxic agents. Because SM acts as a cytotoxic agent, this triggered our interest in amifostine and its analogues as possible prophylactic agents against SM intoxication. Ten analogues of amifostine were synthesized by varying the chain length and also using different substitutions at the sulphur center. These were compared with amifostine as a prophylactic agent for SM in mice.

These compounds were administered intraperitoneally in mice 30 minutes prior to dermal application of SM. The dose of the prophylactic agent was equal to 0.2 LD_{50} and that of SM was 230 mg.kg-1 equal to 28 times LD_{50} of SM. Amifostine and one of its analogues, DRDE-07, gave significant protection. The protection offered by DRDE-07 was better than that of amifostine. Further studies were carried out in mice on the dose response relationship of amifostine and DRDE-07 by administering them orally. Thirty minutes after oral administration SM was applied dermally at varying LD_{50} doses. DRDE-07 was able to protect at its 0.1 and 0.2 LD_{50} doses, while amifostine protected only at 0.2 LD_{50} dose.

INTRODUCTION

Bis (2-chloroethyl) sulphide, commonly known as sulphur mustard (SM) or mustard gas, is an alkylating agent that causes serious blisters upon contact with human skin. SM forms sulphonium ion in the body and alkylates DNA, leading to DNA strand breaks and cell death. Eyes, skin and the respiratory tract are the principal target organs of SM toxicity[1,2]. Several antidotes have been evaluated for reducing the systemic toxicity of SM, but most of the compounds screened so far have given only limited protection. Despite the signing of the Chemical Weapons Convention (CWC) and its subsequent ratification by several countries, the possibility of SM being used clandestinely during warfare or by terrorist groups still exists due to the simple method of SM preparation. Also during the destruction of the stockpiled SM and during the inspections by the Organization for the Prohibition of Chemical Weapons (OPCW) a prophylactic agent for SM may be an important requirement.

The symptoms of mustard exposure are similar to those caused by radiation[3,4], which has led to some studies on the protection offered by radioprotectors.[1] Amifostine or WR 2721 [S-2(3-aminopropylamino)ethyl phosphorothioate], a potent radioprotector, has been evaluated against nitrogen mustard intoxication and showed potential as a prophylactic agent[5-7]. No data, however, are available on the effectiveness of amifostine against SM. The present work was, therefore, initiated in order to develop an effective prophylactic agent for SM, by evaluating amifostine and related compounds.

MATERIALS AND METHODS

Chemicals: Amifostine [S-2(3-Aminopropylamino) ethyl phosphorothioate] and its analogues were synthesized following the method described in the literature. Other compounds were prepared by the procedures adopted from the general synthetic routes described for such classes of compounds. The compounds were characterized by analytical and IR, ^{1}H and mass spectral data.

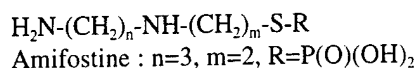
Animals: Randomly bred Swiss female adult mice (25-29 g) were obtained from the animal house of our establishment. The animals were acclimatized for 8-10 days in polypropylene cages on dust free rice husk and fed pellet diet (Amrut Feeds, India) and water *ad libitum*.

Protection studies: Hair from the back of mice was closely clipped using a pair of curved scissors, 16-20 hours prior to the experiments. Freshly prepared solution of test compounds in distilled water was fed using oral feeding needle or injected intraperitoneally (0.2 LD_{50}). The feeding volume was kept constant in all experiments. The animals were fed or injected with the solution of test compounds before thirty minutes. Sulphur mustard diluted in PEG-300 was applied on the hair clipped area of skin. The animals of control groups were fed or injected with equal volume of distilled water at the same time interval prior to SM application. The animals were observed for 14 days for mortality.

Statistical analysis: The data were analyzed by Friedman's repeated measures ANOVA on ranks followed by Dunnett's method for comparing with control. All the analysis were made using Sigma Stat software (Jandel Sci. USA). LD_{50} was determined following method of moving average.[8]

RESULTS AND DISCUSSION

Amifostine and its analogues are S-(ω-aminoalkylamino)alkyl phosphorothioates with following general chemical structure.



$H_2N\text{-}(CH_2)_n\text{-}NH\text{-}(CH_2)_m\text{-}S\text{-}R$

Amifostine : n=3, m=2, $R=P(O)(OH)_2$

We studied several such analogues with varying chain length. While looking for other compounds for potential antidotal activity we examined several derivatives having S-(ω-aminoalkylamino)alkyl chain in their chemical structure. It may be noted that the presence of phosphoric moiety in amifostine reduces the lipophilicity and thereby adversely affects the bioavailability of the molecule in the body. It was therefore presumed that favorable effects might be achieved by replacing the phosphoryl group with some other group that may not only enhance the lipophilicity of the molecule but also help in scavenging SM present in the body. Keeping these basic concepts in mind, we synthesized a series of compounds by changing the phosphoryl group in amifostine such as thiols (R=H) and thioethers (R=alkyl, aryl and heteroaromatic). Mainly two types of substituents were selected; one that will simply influence the lipophilicity of the molecule and the other that besides enhancing lipophilicity may also help in scavenging SM. The first type of substituent include phenyl, tolyl and chlorobenzyl groups while the second type of substituents are N-methyl-imidazolyl and pyridyl groups. Amifostine and 10 of its analogues and related derivatives were synthesized. These compounds were characterized on the basis of their elemental analysis and spectral data.

The preliminary studies showed that two out of all the test compounds, namely amifosine and another derivative coded DRDE-07 (patent filed)[9] showed promising results, which have been the subject of further investigation. Table 1 shows the mortality caused by percutaneously applied sulphur mustard (SM) in i.p. pretreated female mice (-30 min) with amifostine and DRDE-07. The results show less mortality of the animals of pretreated groups compared to control group of animals and a statistically significant protection was observed.

Both compounds were also tested by administration through oral route. Table 2 shows the dose response in terms of per cent protection against SM toxicity of three doses of amifostine and DRDE-07 in mice. The pretreatment time was kept 30 minutes for all the doses of amifostine and DRDE-07. The results of the orally administered compounds are shown in Table 3, which shows significant protection by both the compounds. The data also show that protection given by DRDE-07 is better in comparison to Amifostine.

Sulphur mustard is a well-known cytotoxic agent[2,10]. The toxic effect of SM is mainly due to alkylation of vital biomolecules including DNA. Sulphur mustard induced cytotoxicity is also based on lipid peroxidation occurring to the formation of reactive oxygen intermediates as a consequence of depletion of glutathione (GSH)[1]. Any therapeutic measures should interfere with the mechanism of intoxication of SM. Perhaps amifostine acts through its free thiol (-SH) in scavenging the sulphonium ions formed between the highly reactive SM with vital molecules. Detail studies are underway to understand other possible mechanisms of protective action of amifostine in SM poisoning.

To conclude, the above study brings out amifostine and DRDE-07 as promising compounds effective against SM toxicity. Though earlier studies indicated that amifostine has potential to minimize the toxicity of anticancer drugs, this is the first study to explore the possibility of use of amifostine as an oral drug against SM toxicity. We believe that it has great potential in the pretreatment of SM poisoning. Other related compounds such as DRDE-07 may be better as prophylactic agent against SM and research is in progress.

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KEYWORDS
Sulphur mustard, amifostine, DRDE-07, toxicity, antidotes, prophylaxis

Table 1. LD_{50} and protective efficacy of amifostine and DRDE-07 administered intraperitoneally on sulphur mustard induced lethality in mice.*

Agents	LD_{50}[a] ($mg.kg^{-1}$)	Lethality percentile after SM[b]		
		25th	50th	75th
Control	-	85	100	100
Amifostine	1131	40	60*	80
DRDE-07	336	45	60*	80

*Dose of agents for protective efficacy was 0.2 LD_{50}; Dose of undiluted SM was 230 $mg.kg^{-1}$ (28 times LD_{50}) applied dermally; Statistically significant compared to SM group by Friedman's repeated measures ANOVA followed by Dunnett's method; $\chi^2 = 26.7$; df = 2;
P = < 0.001
[a]For LD_{50} determination 3 to 4 groups were used with each group consisting of 4 mice.
[b]For protection studies 5 mice per group were used. Control group was a pooled data of 29 mice. 25th, 50th and 75th percentiles are the percent of mice dying after 3.5, 7 and 10.5 days.

Table 2. Dose dependent efficacy of amifostine and DRDE-07 administered orally against topically applied sulphur mustard in mice*

	Treatment/Pre-treatment, Dose (LD_{50})	Lethality percentile after SM[a]		
Agent		25th	50th	75th
Amifostine	0.05	0.00	50.0	100

	0.10	0.00	0.00	100
	0.20	0.00	25.0*	50.0
DRDE-07	0.05	0.00	00.0*	69.0
	0.10	0.00	25.0*	69.0
	0.20	0.00	00.0*	25.0

*LD_{50} (oral): Amifostine = 1049 mg/kg and DRDE-07 = 1247 mg/kg; Dose of SM was 155 $mg.kg^{-1}$ (19 times LD_{50}) diluted in PEG-300 and applied dermally; Statistically significant compared to SM group by Friedman's repeated measures ANOVA followed by Dunnett's method; $\chi^2 = 40.6$; df = 6; P = < 0.001

[a]Eight mice per group were used. 25th, 50th and 75th percentiles are the percent of mice dying after 3.5, 7 and 10.5 days.

Table 3. Time dependent efficacy of amifostine and DRDE-07 administered orally against topically applied sulphur mustard in mice*

Agent	**Treatment time (min)**	**Lethality percentile after SM[a]**		
		25th	**50th**	**75th**
Control	- 30	2.5	50	70
Amifostine	0	0	40	90
	- 30	0	10*	47.5
	- 60	10	10*	57.5
DRDE-07	0	0	0*	27.5
	- 30	0	0*	20
	- 60	0	10*	30

*Dose of agents for protective efficacy was 0.2 LD_{50} (Oral LD_{50} of amifostine=1049 $mg.kg^{-1}$ and DRDE-07=1248 $mg.kg^{-1}$); Dose of SM was 155 $mg.kg^{-1}$ (19 LD_{50}) diluted in PEG-300 and applied dermally; Statistically significant compared to SM group by Friedman's repeated measures ANOVA followed by Dunnett's method; $\chi^2 = 57.4$; df = 6; P = < 0.001

[a]Ten mice per group were used. 25th, 50th and 75th percentiles are the percent of mice dying after 3.5, 7 and 10.5 days.

34. EFFECTS OF SODIUM BICARBONATE IN ORGANOPHOSPHATE PESTICIDE POISONING

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INTRODUCTION:

Organophosphate (OP) compounds are widely used as pesticides in many parts of the world. Acute OP pesticide poisoning is a common cause of admission to the clinical toxicology ward of Imam Reza hospital. Insufficient control on the importation, production, storage and safe use of OP pesticides are the main reasons of poisoning (1).

OP compounds have also been manufactured and used as chemical warfare agents. In 1983 & 1984 in Majnoon Island and in the Halabjeh massacre (1988), OP compounds were used as nerve gases by Iraqi troops. Analysis of the environment, urine and blood samples of victims proved Tabun in Majnoon Island and Sarin in Halabjeh (2,3). Terrorist attacks in Matsomoto (1994) and in the Tokyo subway (1995) by Sarin nerve gas caused 18 deaths and thousands of casualties (4,5).

The very well known antidotes of organophosphate (OP) poisoning are atropine sulphate and oximes (6-8). Atropine counteracts the muscarinic effects of OP poisoning, but the therapeutic effects of oximes are controversial and are not the same in different OPs (9-12). In addition, the high cost of pralidoxime and the hepatotoxicity of obidoxime, particularly with high doses, have urged the clinicians and scientists to search for more additional effective treatment (13).

Palacio studied the effects of sodium bicarbonate (NaHCO3) in OP pesticide poisoning on experimental animal models with a positive outcome (14). It was thus aimed to study the effects of $NaHCO_3$ in treatment of patients with acute OP pesticide poisoning.

PATIENTS AND METHODS

The patients (aged 14-60 years) were admitted to the hospital between April 1996 and March 1999 with a history of intentional oral ingestion of a known OP pesticide with ingestion – admission interval of less than 6 hours and revealed clinical features of moderate to severe poisoning were studied. The patients were divided into two groups:

The control patients received atropine (A) and the other received treatment as usual. Atropine sulfate was administered by I.V. to control the muscarinic effects and to induce mild to moderate atropinization (dry mouth, tachycardia, flushing and mydriasis) within 30 minutes. Atropine infusion was continued based on clinical responses to maintain the atropinization. Atropine was tapered based on clinical improvement.

The test group was treated just as the controls, but included was an infusion of sodium bicarbonate (AB) initially 3mEq/kg in one hour, followed by 3mEq/kg in 23h and the same amount every day until recovery/death. Arterial blood gas and pH (ABG) were estimated on admission and at certain intervals by a blood gas analyzer (AVL-99). Acetylcholinesterase (AChE) activity was estimated in red blood cells by the modified Ellman method (15). Clinical and paraclinical findings including AChE activity were recorded on pre-designed forms. The results were analyzed by the Chi-square and Student-t tests using a statistical package for social sciences (SPSS). The results are shown as mean and standard deviations.

RESULTS

Over the three years of study, 50 patients (27M and 23F) were studied in the two groups. Age, sex, weight and vital signs of the patients in each group are summarized in Table 1.

There were no statistically significant differences between the groups on the OP types, AChE activity (385± 346 U/L in the test and 428 ± 435 U/L in the controls), arterial blood pH (7.36± 0.12, and 7.32± 0.12, respectively) and initial atropine dose (48.0± 39.1 and 53.7± 35.8 mg, respectively) required for atropinization on admission. However, on admission clinical findings, only cyanosis and respiratory arrest were significantly higher ($p<0.05$) in the AB group (Table 2).

There were also no statistical significant differences on AChE activity, clinical severity and ABG during treatment, hospitalization days and ICU therapy between the groups.

Maximum arterial blood pH and $NaHCO_3$ in the test group were 7.47± 0.3 and 24.4± 2.4 m Eq/L, respectively, which were significantly higher than the controls (7.37± 0.8 and 19.5 ± 4.5 mEq/L, respectively, $p<0.01$) as shown in Table 3. Three patients of the control and only one of the test group died. However, the differences in arterial blood pH and $NaHCO_3$ were not statistically significant.

DISCUSSION

The severity of intoxication on admission based on clinical findings except for the cyanosis and respiratory arrest, which was higher in the AB group, were similar. Paraclinical findings on admission were also similar in the groups. Thus, the test group had similar and even higher severity of intoxication on admission. Sodium bicarbonate was also used to correct the metabolic acidosis. However, the amount of sodium bicarbonate administered was not enough to induce alkalosis as judged by the arterial pH and bicarbonate of the two groups during treatment. Even the maximum arterial pH and bicarbonate which were significantly higher in the AB group, were lower than the results achieved by Placcio in his animal experiment (14).

It is thus recommended to administer more sodium bicarbonate to achieve an arterial pH of 7.50 to 7.55. Alkalinization of the blood to pH of more than 7.50 by sodium bicarbonate facilitates destruction of OP molecules. Moreover, OPs are ester of phosphoric acid and hydrolysis of the molecules increase with higher pH (16). However, different chemical structures of OPs may reveal different stabilities in acid solution and react differently in alkali solution. Dimethoate, methyl parathion, malathion and trichlorfos in particular were more stable in acid solution whilst diazinon was less stable in acid solution (17). This could suggest that sodium bicarbonate therapy might be successful in the management of intoxication with some OPs like dimethoate, methyl parathion, malathion and dichlorfos. It is thus very important to study the effects of sodium bicarbonate in each OP separately.

CONCLUSIONS

The sodium bicarbonate doses used in this study were not big enough to produce significant alkalinization. Due to the different chemical structures of OPs, and their different stabilities in acid solution, further study is recommended with higher doses of sodium bicarbonate in each OP separately.

ACKNOWLEDGEMENTS

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FIGURES AND TABLES

Table 1. Patients and their Vital Signs on Admission

	Control (Atropine)	Atropine + Bicarb.
1. Age (year)	24.9± 10.8	23.5 ± 9.7
2. Sex	15M± 11F	12M ± 12F
3. Weight(kg)	63.4 ± 14.5	59.8 ± 12.3
4. Systolic B.P.(mmHg)	113.6± 26.0	110 ± 12.1
5. Diastolic B.P. (mmHg)	67.1 ± 16.7	71.3 ± 13.2
6. Pulse (min)	90.5 ± 25.0	86.8 ± 25.8
7. Resp. rate(min)	20.3 ± 8.5	22.0 ± 9.9
8. Temperat. (C)	36.9 ± 0.7	37.2 ± 0.3

Table 2. Clinical Findings (%) on Admission and During Treatment.

Clinical Findings	Control (Atropine)		Atropine + Bicarb	
	On Admis.	Treatment	On Admis.	Treatment
1. Nausea/ Vomit	76	0	70	12
2. Diarrhea	26	15	20	8
3. Abd. Pain	53	11	45	20
4. Miosis	84	0	62	0
5. Hypersecr	69	40	76	8
6. Sweating	34	0	41	0
7. Rale/crackle	26	0	20	4
8. Pulm. Edema	11	0	8	0
9. Cyanosis	7.5	0	16*	4
10. Resp. arrest	7.5	7.5	20*	4
11. Agitation	30	3.7	33	12
12. Confusion	34	7.5	25	4
13. Coma	46	11	34	8
14. Twitching	42	3.7	37	12
15. Convulsions	8	0	12	0
16. Cardiac Arrhyt.	0	0	8	0
17. Cardiac Arrest.	7.5	3.7	0	0

*P<0.05

Table 3. Arterial pH, Bicarbonate and Gases

ABG	Control (Atropine)		Atropine + Bicarb	
	On Admis:	Treatment	On Admis	Treatment
pH	7.3 0.1	7.32 0.05	7.36 0.1	7.46 0.15
pH(max)	-	7.37 0.08	-	*7.47 0.03
Bicarb	19.7 3.9	17.2 3.8	18.1 4.0	20.5 5.8
PO2	93.9 45.3	92.1 42.3	82.4 28.5	87.4 37.8
PCO2	34.7 7.4	35.2 8.5	34.8 7.0	36.7 5.9

*P<0.01

35. A REVIEW OF INFECTIONS AMONG IRANIAN COMBATANTS IN THE IRAN-IRAQ WAR

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INTRODUCTION

The Iraqi army in 1980 invaded Iran in a war which lasted about eight years. From 1984, Iraqi armed forces used chemical and biological weapons in massive attacks against Iran. Several institutes and universities used the opportunity to study the different aspects of infections among the Iranian soldiers. (1) We studied the different infectious agents among Iranian combatants and to compare our findings to those found by our colleagues and also to those found during the past wars in the world.

Wounds caused by high velocity missiles are characterized by extensive tissue destruction and often there is deep penetration of metal fragments, dirt and debris into the wounds. Invasion of the tissues by many organisms invariably occurs and even with availability of potent antibiotics, wound infections of this type are still regarded as a major treatment problem in combat—injured personnel. (2, 3)

MATERIAL AND METHODS

Shiraz is a city located in Fars province and is about 400 miles from the Iran—Iraq border. During the Iran—Iraq conflict, the war casualties were first collected by frontline paramedics and transferred to ambulance stations about one mile away from here the victims were transported by surface to emergency care stations about 5 miles behind the frontline. With two general surgeons stationed there, emergency resuscitation was accomplished including total blood transfusion and intubation of chest tubes and infusion of antibiotics and dexamethasone. From emergency care stations the patients were transferred by helicopter to the base or rear hospitals located approximately 100—200 miles behind the line of campaign. Primary definitive exploration was performed in these local centers by surgeons and subsequently the patients were transferred to major provincial centers for exploration. A number of patients were evacuated from these head centers directly to major cities to have their primary definitive exploration in the latter surgical facilities.

Bacteriological studies were done in 126 severely war-injured soldiers from November 1985 to February 1q86 in the Nemazee-University Hospital in Shiraz, The war injured soldiers were young males with a mean age of 19.5 years. They had received battle field first aid treatments including administration of I.V antibiotics (Keflin & Centamicin) in the frontline emergency stations and underground hospitals.

Nine patients with penetrating colonic injuries and twenty patients with missile head wounds had been on chloramphenicol and crystalline sodium penicillin G. during admission at the Hospital The time from injury to admission at the Hospital was from 48 to 72 hours.

Shell fragments were the most common cause of injury. The wounded soldiers were all injured with multiple high velocity missiles. Wound cultures were obtained with sterile cotton swabs from the edges of the most severe wound of the casualties before debridement. The anatomical sites of cultures were different.

Specimens for isolation and identification of bacteria were cultured on thioglycollate, EMB and blood agar. Colonies were subcultured on differential media for further identification according to classical method (4). Antibiotic sensitivity tests were performed by disc method according to Kirby-bauer diffusion method.

RESULTS

The isolated bacteria from different sites showed the presence of Pseudomonas aeruginosa (33.34%), Klebsiella (14.29%), Enterobacter (14.29%), E. coli (11.90%), Proteus (9.5^2%), Acinetobacter (9.52%) and Staph aureus (7.14%) (Table 1).

Table 2 demonstrates the resistance of Pseudomonas aeruginosa for each of antibacterial agents. It shows that Pseudomonas aeruginosa was resistant to ampicillin, cephalosporins, chloramphenicol Kanamycin, trimethoprimesulfonamide and tetracyclines.

Table 3 shows the resistance rate of 117 gram—negative rods isolated from soldiers wound during Iran-Iraq war we see that gram negative bacteria (117 isolates) were resistant to ampicillin (100%) cephalosporins (97.43%), chloramphenicol (89.74%), gentamicin (46. 15%) Kanamycin (66.66%), tetracyclines (93.16%), Trimethoprimsulfonamide (92.30%), and amikacin (8.54%).

DISCUSSION

A projectile at a very high velocity can not only penetrate the tissue, but release a large amount of kinetic energy to the surrounding tissues. The damage comprises the destruction of tissue due to stretching of nerves and large blood vessels, and actual fracture of bones —several centimeters away (2). So, war wounds characteristically are lesions in which tissue injury is extensive and universal contamination by bacteria present in the environment active proliferation of bacteria may be demonstrable in muscles away from the wound tract as early as six hours after injury (3). Infection is thus an ever present possibility and it continues to be a threat until healing is complete. The organisms involved included those from the skin and clothes as well as those from soil, dirt and debris that penetrate the wounds

Bacteriology of War Wounds

The microbial flora of war wounds have been under constant evaluation. Fleming published the first comprehensive bacteriological evaluation of war wounds incriminating Clostridium species and Streptococcus pyogenes (6). The organisms found in wounds during World War 1 were roughly divided into three groups:

1. sporulating microbes of fecal origin, consisting chiefly of gas producing bacterial species Clostridium welchi (perfringens), Clostridium oedematis maligni, Clostridium histolyticus, Clostridium sporogenes, Clostridium tertius, Clostridium septicum and Clostridium tetani
2. nonsporulating microbes of fecal origin, including the enterococci and
3. bacteria of the colon-typhoid group. (3), pyogenic cocci, consisting chiefly of the Staphylococci and Streptococci (7).

Pettit showed, in his survey of World War I wounds, that the initial infection was due to Clostridium welchi and that after approximately one week, the majority of wounds yielded Streptococcus pyogenes (8). During subsequent wars, the microbial flora shifted to the pathogenic cocci: Staphylococcus aureus and Streptococcus pyogenes. The apparent decrease in the invasive power of Clostridium species can be attributed to immediate debridement of the wound. About 30% of traumatic wounds May be contaminated by C. perfringens, but few of them develop the clinical picture of gas— gangrene (9). Clinical gas gangrene develops when the oxidation reduction potential of the tissue is lower, and the Clostridial spores are converted to vegetative form. Improved methods of wound care and the use of antibiotics decreased the incidence of gas— gangrene from 12% in World War 1 to 0.3% to 0.8% in World War 11 and to 0.08% in the Korean War(9). In Vietnam War only 22 cases of gas—gangrene were reported.

Gram negative infections of War Wounds considered rare during World War 1 but became more evident during World War 11 when infections by coliform bacteria and, to a lesser extent, Proteus and Pseudomonas organisms were reported (10). The infection by gram negative bacilli also became predominant during the Korean (11). Arab-lsrael (12) and Vietnam (13) Wars.

This study also showed that gram negative bacilli, e.g., Pseudomonas aeruginosa (33.34%), were the most common bacteria from combat wounds in the Iran Iraq war. The shift from gram positive to gram negative bacteria is thought due to the sulfonamides and penicillin administration during the latter part of World War II (3). Penicillin has lowered the incidence of Clostridial infections, gas gangrene and infection by hemolytic Streptococci. Lindberg incriminated Staphylococcus species, Streptococcus species and the Enterobacteria as causative agents of infection in Wounds, during the Korean war (11).

A review of laboratory data and bacteriological studies collected at the 249th General Hospital (during the Vietnam war) revealed that the organisms most frequently isolated from wounds were Staphylococcus aureus. Pseudomonas, aeruginosa and E. coli (14). The common isolated bacteria from war wounds in the Arab—Israel war were Pseudomonas, Klebsiella and E. coli. (12)

Pseudomonas aeruginosa was the most common single pathogen in our study. It is compatible with Heggers (15) and Tong (13) in Vietnam and Simchens' studies (12) in the Arab—Israel war.

COMMENTS

Experiences during World War 1&11 and their comparison to the Korean, Vietnam and Arab-Israel wars showed that penicillin is effective in decreasing the incidence of gas-gangrene and eliminating infections by hemolytic streptococci. Thus, one of the penicillins or cephalosporins (e.g., cephalothin) for coverage of penicillin-resistant staphylococci should be administered with the fluids at the level of the battalion aid stations or underground hospitals in the front lines after wounding as possible.

Because of the frequency with which gram-negative bacteria were isolated from wounds, adequate antibiotic coverage for these organisms should also be included in the drug regimen. Antibiotic therapy should be started prior to surgery as manipulation of these high velocity missiles war wounds invariably causes transient bacteremias. Gentamicin and clindamycin in penetrating colon injuries with projectiles might prevent some of the infections associated with this particular risk factor. Chloramphenicol in combination with either ampicillin or crystalline sodium penicillin C should be given for patients with missile head wounds.

The use of antibiotics in military surgery is to prevent infection after proper wound debridement or to treat already established infection originating in necrotic tissue. For this reason they are not intended to replace immediate surgery, complete excision of devitalized tissue or the surgical drainage of pus. After evaluation of the patients in the Hospitals away from the front lines, antibiotic regimen must be changed according to antibiotic sensitivity pattern of the bacteria responsible for nosocomial infection of the hospital.

Because, with most of the late-onset infections, the problem is that of nosocomial infections in these combat injured patients. Pseudomonas (the most common causative of war wounds infection) and Klebsiella were the most common cause of death in war casualties. These two organisms are known to be responsible for nosocomial infection. Thus. strategies including controlled antibiotic usage must be designed for control of hospital-acquired infection. The clinically isolated bacteria were highly resistant to aminoglycosides (e.g, 46.15% for gentamicin) except for amikacin (8.54%). Thus, it is recommended that amikacin be used instead of gentamicin in hospitals.

This strategy will decrease the gentamicin resistance, due to decline in the selective pressure exerted by gentamicin on the gram—negative bacilli. Then, the susceptible population of bacteria will be reestablished, and the incidence of aminoglycoside resistant bacteria declined. It must be noted that unrestricted use of amikacin has not led to s concomitant increase in amikacin resistance in gram negative bacilli.

The most common isolated bacteria from war wounds in World War 1 & 11, the Korean, Vietnam, Arab-Israel. and Iran-Iraq wars.

Author	War	Organisms
Fleming	World War 1	Clostridium species Streptococcus pyrogenes
Pettit	World War 1	Clostridium welchii Streptococcus pyrogenes
Miles	World War II	Staphylococcus aureus Streptococcus pyrogenes
Linberg et al	Korea	Staphylococcus specied Streptococcus species enterobacteria
Heggers et al	Vietnam	Pseudomonas aeruginosa
Tong	Vietnam	Pseudomonas and Proteus
Simchen et al	Arab-lsrael	Pseudomonas Klebsiella E. coli
Nabipour et al	Iran-lraq	Pseudomonas aeruginosa Klebsiella Enterobacter

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FIGURES AND TABLES

Table 1. Bacterial Species isolated from 126 war wounds. Three patients had two different organisms and three wound cultures showed no bacterial growth.

Bacteria	No. Species	% Isolates
Pseudomonas	42	33.34%
Kiebsiella	18	14.26
Enterobacter group	18	14.26
E. coil	15	11.90
Acinetobacter	12	9.52
Proteus	12	9.52
Staph	9	7.14

Table 2. Percent resistance rate of pseudomonas aeruginosa for each of antibacterial agents.

Antibiotic	No. Resistant Isolates	%Resistance Rate
Ampicillin	42	100
Cephalosporins	42	100
Chloramphenicol	42	100
Gentamicin	21	50
Trimethoprim	42	100
Kanamycin	42	100
Tetracyclines	42	100

Amikacin	3	7.14

Table 3. Percentage of resistance rate of 117 gram negative bacteria isolated from wounds during Iran-Iraq war.

Antibiotic	No. of Resistant Isolates	% Resistance Rate
Ampicillin	117	100
Cephalosporins	114	97.43
Tetracyclines	109	93.16
Trimethoprim	108	92.30
Chloramphenicol	105	89.74
Kanamycin	78	66.66
Gentamicin	54	46.15
Amikacin	10	8.54

36. DETERMINATION OF AFLATOXINS BL AND ML IN CHICKEN LIVER BY HPLC

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ABSTRACT

Mycotoxins are toxic or carcinogenic secondary metabolites produced by fungi on agricultural commodities. The presence of mycotoxins in the food and feed stuffs is a result of a complex series of interaction among the causative fungi, the contaminated products, the various environmental factors and the intoxicated host. aflatoxinis produced generally by Aspergillus species that contaminates food and feed. Epidemics due to these toxins have occasionally killed many domestic animals.In this work 100 samples of chicken liver collected from poultry producers and liver were analyzed to determine aflatoxin Bl and Ml both qualitatively by TLC and quantitatively by HPLC. The samples were extracted with dichloromethane and a column of silica gel was used to remove fats and other impurities. Then it was eluted by dichloromethane and acetone in the ratio (4:1) solvent was evaporated and the residue was dissolved in known amount of dichloromethane for further experiments. Preliminary results by TLC showed that about 43 % of the samples were contaminated with aflatoxins and then were separated and quantified by HPLC. The results on HPLC showed that the highest levels of aflatoxin Bl and Ml were 0.71% and 1.1 µg/100 grams of samples respectively. On the other hand the minimum amount of aflatoxin B 1 and Ml which were detected by TLC were 0.11 and 0,14 µg/100 grams of samples as judged by HPLC. According to the previous reports these levels of aflatoxins are considered as safe but should be noted that the production of aflatoxins may be accelerated by improper production and handling of feeds.

INTRODUCTION

The impact of mycotoxins on human and animal health is well recognized. Mycotoxin entry to the human and animal dietary systems is mainly by ingestion takes place and exhibit a wide range of adverse biological effects and individual mycotoxin can be mutagenic, carcinogenic, embryo toxic, teratogenic or estrogenic. Establishing a causal relationship between mycotoxins exposure and human disease is complicated(l). Mycotoxins are chemical compounds produced by variety of fungi that can cause illness in human and animals. The adverse effects of fungal products have caused mass poisoning in both man and animals in many countries. As far as public health problems are concerned, aflatoxin is well known as one of the most important environmental toxicants, since its potent hepato-toxicity has been demonstrated in various experimental animals and its natural occurrence in cereals and grains has been shown by chemical analysis (2, 3). The presence of mycotoxin producing fungi have been well demonstrated as a natural pollutant in several plant products including cereals, grains and foodstuffs in many countries of Europe, Russia, USA, Canada, and several Asian countries (4).

The aim of this investigation was to determine qualitatively and quantitatively the aflatoxin Bl and aflatoxin Ml in chicken liver by high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Liver tissues were collected from local poultry producers. aflatoxins Bl and Ml were obtained from Sigma CO. The HPLC eluent solvent and other chemicals were purchased from Merck.

The liver samples were homogenized and then to the 100 grams of homogenized liver 10 mL of 20 % citric acid were added and mixed well then 200mL of dichloromethane were added and kept in automatic shaker for about 30 minutes .The mixture was filtered and the filtrated materials were evaporated under vacuum (5). Then column was packed with silica gel and the extracted materials were poured to the top of the gel in column and eluted with hexane to remove fats. Other impurities were removed by a mixture of hexane , ether and ,acetonitrile in the ratio of 1 : 3 : 6 respectively. Then column was eluted with a mixture of dichloromethane and acetone. Aflatoxins were obtained after evaporation of the organic solvent and again the sample was dissolved in a known volume of dichloromethane and it was prepared for thin layer chromatographic and high performance liquid chromatographic determination.

Thin layer chromatography has been a convenient and simple method for analysis. The solvent system employed was isopropanol, acetone and chloroform (8 : 10: 82) and aflatoxins Bl and Ml were detected qualitatively under UV after sprayed with 50 % sulfuric acid . For quantitatively determination we employed HPLC which was equipped by UV-detector at 244nm and the mobile phase was actonitrile, methanol and water in the ratio of 45:25:30 respectively

RESULTS AND DISCUSSION

With regards to the method validity those samples which were positive in TLC were analyzed by HPLC and the results are shown in Table 1.

The HPLC chromatograms of standard aflatoxin Bl and aflatoxin Ml are shown in fig. 1.In fig 2. the HPLC chromatogram of a contaminated sample is shown. In this study 100 liver samples were collected from different local poultry and were analyzed for aflatoxins Bl and Ml contamination by TLC and HPLC and emphasis was given to HPLC method. The impact of mycotoxins on human and animal health is well understood and to obtain a clear view on food products it is worthy to carry out the best sensitive and accurate method of analysis because aflatoxin B 1 is recognized as a potent toxic carcinogenic substance (6, 7) and its metabolite aflatoxin M! in the biological fluids.

The results from this investigation showed some contamination but it is not in the range of high toxic level as shown in Table 1. Results on HPLC showed that the highest levels of aflatoxins Bl and Ml were 0.71 and 1.1 μg/100 grams of samples respectively. On the other hand the minimum concentration of aflatoxin Bl and Ml which were detected by TLC were 0.11and0.14ug/100gramsofsamplesasjudged by HPLC. According to the previous reports these levels of aflatoxin Bl and Ml are considered as safe but it should be noted that the production of aflatoxins may be accelerated by improper production and handling of feeds.

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FIGURES AND TABLES

Figure 1. HPLC Chromatogram of standard aflatoxin Bl and Ml

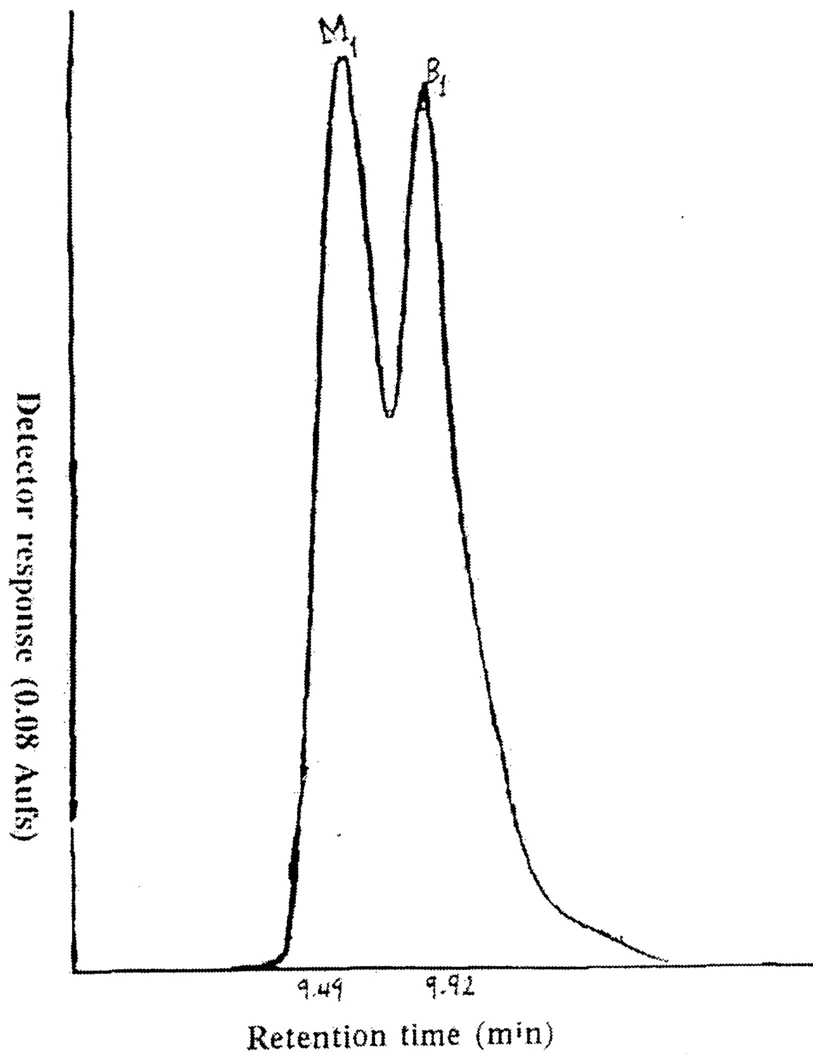

Figure 2. HPLC Chromatogram of contaminated sample.

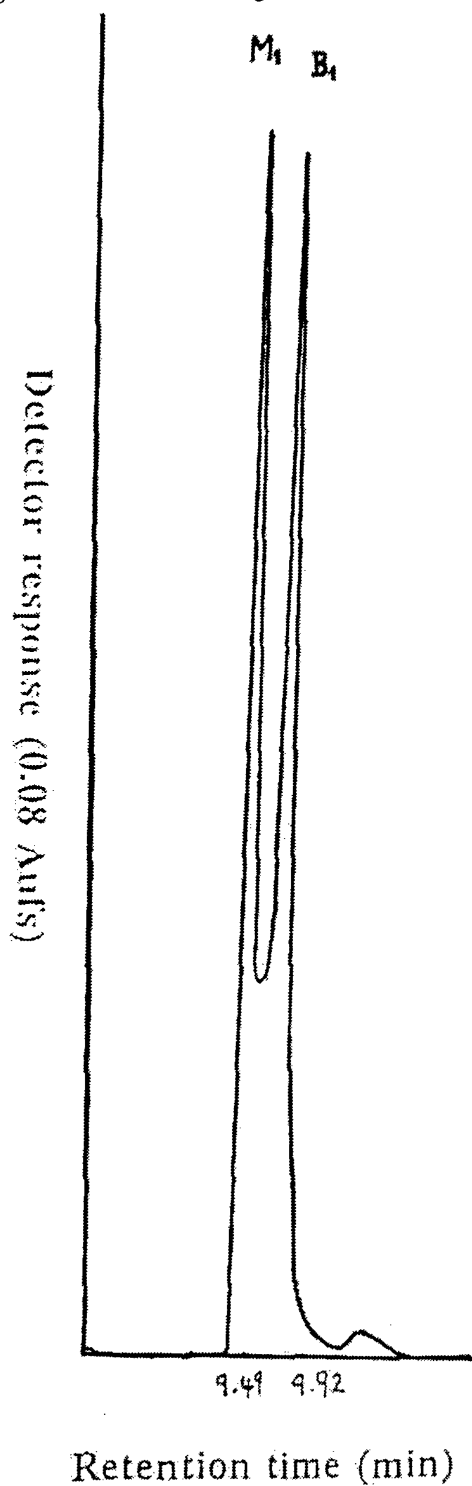

Figure 3. Column chromatogram.

Table 1: Liver samples were analysed for Aflatoxin Bl and Ml by HPLC

Aflatoxin	*Total samples*	*Positive samples*	*Maximum Conc. μg/100r*	*Minimum Conc. μg/100g*	*Mean conc. of positive sample μg/100g*	*SD*
Bl	100	43	0.71%	0.11	0.46	0.17
Ml	100	43	101%	0.14	0.6	0.28

37. TOPICAL TREATMENT WITH IODINE REDUCES SKIN TOXICITY CAUSED BY SULFUR MUSTARD

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INTRODUCTION

Sulfur mustard (SM, mustard gas) is a potent blister agent employed as a chemical weapon in various conflicts during the 20th century (Mellor, et al. 1991). It is a potent alkylator and highly cytotoxic agent in both humans and animals (Dacre & Goldman 1996). Skin exposed to SM develops erythema followed by edema, vesicle and blister formation, ulceration, necrosis and desquamation (Smith, Graham, et al., 1995). It is accepted that the powerful alkylating activity of SM (Ludlum, et al. 1994) results from its conversion, in aqueous solution, to the highly electrophilic ethylene episulfonium derivative (Wormser 1991) which can be neutralized by nucleophilic agents. It has been proposed that protection against SM can be achieved by glutathione derivatives (Lindsay & Hambrook 1998). In spite of having some beneficial effects, these agents were not efficacious enough to be used as antidotes. Additional agents such as arginine analogs (Sawyer 1998), calcium channel blocker (Mazumder, et al. 1998), niacinamide (Meier & Johnson 1992) and its combination with promethazine and indomethacin (Yourick, et al. 1995) had a weak protective effect as post exposure treatment against SM in experimental animals although some of them were beneficial in *in vitro* and *in vivo* systems particularly as prophylactic treatment.

Iodine and iodophors like povidone iodine are widely used as antiseptic agents. Since their bacteria-killing effect stems from the oxidizing activity of iodine, our primary approach was to employ this characteristic to chemically neutralize SM by oxidation of its sulfur atom to form the less active sulfoxide form. Preliminary studies of our laboratory have shown that post-exposure treatment with povidone iodine protected against SM at interval of 10 min between exposure and treatment whereas at longer intervals the treatment was less effective (Wormser, et al. 1997). Our experience showed that the formulation of iodine plays a crucial role in its counter-irritating activity. Thus, in the present study we have developed a formulation capable of solving molecular iodine in an aqueous environment. The present study demonstrates the potent protective effect of the new iodine formulation against SM-induced skin lesions. The gross- and histopathology of guinea pig skin at different time intervals between SM exposure and iodine treatment were quantified.

MATERIALS AND METHODS

Backs of haired guinea pigs were shaved 24 hours prior the experiment. The animals were anesthetized by 30 mg/kg pentobarbital sodium ip. Backs were cleaned with wet soft white paper and let to dry out before the beginning of experiment. Six sites (three on each side) of each back were exposed to 1μL (1.2 mg) SM. One mL liquid iodine (Merck) preparation (otherwise indicated 2% in tetraglycol: water 1:1) was applied on three exposure sites of each animal while the other 3 SM-exposed sites of the animal remained untreated. Iodine was applied into wells that constructed by the following procedure. A plastic tube cover (inner diameter of 1.7 cm) was cut to form open-ended cylindrical well and a thin layer of commercial silicon sealing ointment was applied to one edge of the well. The well was then attached to the animal back so liquid inside the well did not leak out. Wells were constructed before exposure while SM was applied on the center of the well. Iodine was applied into the well 15 and 30 min after SM exposure. In additional series of experiments the well system was replaced by 3 layers of gauze pad (1.5x1.5cm) soaked in iodine solution (2% I_2 in tetraglycol) applied on site of exposure. Otherwise indicated the iodine preparation was left on the skin for 2 hours. In the end of the procedure the liquid iodine was sucked out and well was removed from the skin. In the end of experiment the animals were sacrificed by 100 mg/kg pentobarbital sodium ip. Their backs were photographed (together with a ruler) by Kodak 260 digital camera and gross pathology was assessed by ulceration area of each exposure site.

RESULTS AND DISCUSSION

Gross pathology evaluation of series of experiments revealed statistically significant reduction of 91% and 84% in the ulceration area at intervals of 15 and 30 min between exposure and treatment, respectively

(Table 1). The vehicle of iodine formulation had some protective effect (32% reduction) but was not statistically different from the exposed, untreated sites. It is also shown that iodine applied by the well system is more efficacious than by the gauze pad procedure (Fig. 1) presumably because the former has bigger reservoir of iodine than the latter.

Numerous iodine formulations were tested but non of them was proved to be as efficient as that containing TG iodine solvent. The superiority of the present iodine formulation stems from its ability to solve molecular iodine (I_2) in an aqueous environment. Molecular iodine (I_2) is practically water insoluble unless iodide (sodium or potassium salts) is present in the solution to form the water-soluble ion I_3^-. Molecular iodine can be dissolved in organic solvents such as ethanol or polyethyleneglycol-400 (PEG-400) but presence of water precipitates the iodine, thus iodine tincture (which contains ethyl alcohol and water) must also contain iodide to form I_3^- for proper dissolution. Experiments carried out in our laboratory showed that iodine tincture had much weaker protective effect than the iodine formulation described in the present study (data not shown). A possible explanation is that the negatively charged I_3^- poorly penetrates through biological membranes and barriers, thus, reducing its efficacy as counter-irritant. However, the TG-containing solvent system that solves I_2 without addition of iodide, thus keeping the molecular iodine in its non-charged form i.e. I_2, might be more penetrable and stronger oxidizer than the negatively charged I_3^-, thus, would be more efficient in its counter-irritating activity. This explanation may also be applicable for the fact that the TG-containing formulation is superior over the iodide-containing formulations against thermal burns and in its bactericidal effect (data not shown). This assumption needs, of course, to be experimentally proved by physico-chemical and biochemical experiments.

Whatever is the reason for the activity of the iodine formulation, the main issue to be addressed concerns the mechanism of the protective action of iodine. The fact that iodine does not chemically inactivate SM was experimentally confirmed (data not shown) is evidence that the protective effect of iodine stems from epidermal/dermal processes affected by iodine. Moreover, the fact that post exposure treatment with iodine is effective also against thermal burns (Wormser 1998) further demonstrates that the antidotal activity of iodine results from cellular events occurring in the skin.

There is increasing evidence that exposure to irritants is associated with the trend of tissue to undergo programmed cell death, namely, apoptosis. Apoptotic cells were demonstrated in cultured keratinocytes (Rosenthal, et al. 1998), endothelial cells (Dabrowska, et al. 1996) and thymocytes (Hur, et al. 1998) after exposure to SM or its derivatives. In vivo studies have shown the appearance of apopatotic cells in SM-exposed skin of weanling pigs (Smith, et al. 1997). The apoptotic process composes a variety of biochemical reaction of which the activity of the cysteine proteinases, caspases, plays an important role (Asahi, et al. 1999). It is hypothesized that iodine exerts its protective activity by inhibition of apoptotic processes, namely, by oxidizing sulfhydryl group of either the active site of caspases or other functional proteins or peptides crucial for apoptosis. This hypothesis is currently under investigation.

Whatever the mechanism of iodine-induced protection is, the present study demonstrates the usefulness of the iodine formulation as a potent antidote against skin lesions caused by SM. It is proposed that this type of topical preparation can be used as a counterirritant at emergencies under both military and civilian circumstances.

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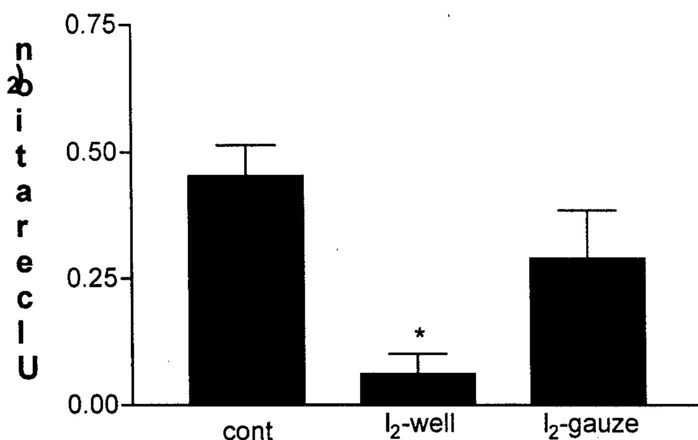
KEYWORDS.
Mustard, sulfur mustard, blister, iodine

FIGURES AND TABLES

Figure 1. Effect of different routes of iodine application on SM skin toxicity.



Guinea pigs were exposed to 1.2 mg SM (1_l) and were topically treated with iodine using two procedures, the well system (n=9) and gauze pad (n=9) as described in Materials and Methods. Ulceration area was measured 2 days following exposure. Iodine was applied 15 min after SM exposure. Control indicates SM-exposed skin without iodine treatment (n=30) Results are expressed as mean ±SE using the Kruskal-Wallis test and Dunnett`s multiple comparison post test for statistical evaluation of the differences between the different routes of exposure and control group. * $p<0.01$

Table 1. Gross pathology effect of iodine against SM-skin toxicity.

Guinea pigs were exposed to 1.2 mg SM (1μL) and were topically treated with iodine as described in Materials and Methods. Ulceration area was measured 2 days following exposure. The time intervals between exposure and treatment were 15 (n=9) and 30 (n=18) min as indicated. Control indicates SM-exposed skin without iodine treatment (n=57) and Vehicle represents iodine vehicle (tetraglycol: water 1:1) applied 15 min after SM exposure (n=9). Results (expressed as percent of control) are mean ±SE using the Kruskal-Wallis test and Dunnett`s multiple comparison post test for statistical evaluation of the differences between the experimental groups.
*** $p<0.001$ at comparison between 15 and 30 min interval and cont.
$p<0.05$ at comparison between 15 min interval and vehicle.

Treatment	Ulceration area (% of control)	SE
Control	100	0
15 min	8.4 ***	0
30 min	15.4 ***	0
Vehicle	0	15.5

39. MASS CASUALTY MANAGEMENT IN THE EVENT OF A CHEMICAL DISASTER

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INTRODUCTION:

Major chemical disasters are mainly associated with accidents, which could be due to either chemical process malfunctioning or human induced activities at storage sites or during transportation of chemicals. Some of the accidents are so sudden that there is no warning for workers or other people in the neighbourhood. As a result, the accidents cause loss of lives property and employment. They also bring about trauma for many people, who may be maimed or injured and their families over many years.

Frequency and intensity of chemical disasters may be controlled but it is very difficult to prevent them all together. When the disasters occur, particularly where mass casualty may take place, there are actions that may be taken to reduce loss of lives and property. Surviving victims require first aid, treatment and counselling. All these activities will require expert manpower and infrastructure and high costs. This is exemplified by the shelling of Ina oil refinery in Croatia about 27 times during the war between 1991 and 1995. Approximately 90,000 m^3 of oil, by-products and other chemicals spilt into rivers Kupa and Sava, thus bringing about adverse effects on the water quality. A total costs of US$65 million was incurred for restoration of the damage caused (Billege et al, 1999).

The purpose of this paper is to address action to minimize chemical disasters, mobilization of resources when the disaster occurs, and post-disaster care for victims and relatives.

PRECAUTIONARY ACTION

Precautionary measures can reduce chemical disasters significantly. The measures include, among others, construction designs, precautionary equipment, monitoring, personnel manifest, awareness and effective expertise. Such expertise is clearly demonstrated by the Japanese authorities response to the terrorist nerve gas attack on a Tokyo sub-way by Aum Shinrikyo Sect in March 1990. Although 5000 people were injured in the attack, only 12 died (Suzuki et al 1995). A larger number would have died if the Japanese authorities did not have sufficient expertise and equipment.

The construction designs for transportation and storage facilities need to take into account appropriate construction materials for the facilities to avoid leakage. The storage and transport facilities should be fitted with warning devices, which are sensitive enough for signals of the slightest precursors of the accidents in order to contain the situation before the accidents occur. Sufficient escape facilities need to be provided to avoid people being trapped in the buildings in case of accidents. There should also be sufficient fire extinguishers and switch off facilities located in strategic areas such that most authorized people may take appropriate action should there be any trigger of possible accident.

The facilities need to be regularly monitored and maintained. In fact, there is need for routine and major maintenance schedules. There is also a need to keep a manifest of the occupants of the facilities, with as many details as possible for ease of identification. This is needed in case of accident after which some of the victims may be unconscious or unable to identify themselves.

Strict surveillance needs to be in place to ensure that penetration of any strange persons including saboteurs into the facilities is avoided. In case of detection of any saboteurs, there should be adequate means for arresting them with minimal interference with the operations of the facilities.

The foregoing disasters are internally induced; externally induced disasters will require different precautionary measures including seeking expertise from specialized institutions in such fields as earthquakes, volcanicity, espionage, bombing etc. in order to take appropriate action in time.

The aforesaid precautionary measures can only be effective if the workers and the residents are fully aware of the dangers associated with any chemical disaster. The workers should not only be made aware of the dangers of accidents and the location of safety facilities but should also be very well trained in the fields of their specialization. In addition there must be a code of ethics in place to ensure that the workers are disciplined in safety matters.

POST-DISASTER ACTION

Despite all precautionary measures within human capability, chemical disasters will still occur. When they occur, a number of measures need to be put in place. These include, among others, early and follow-up measures.

EARLY MEASURES

In the event of any chemical disaster, the following damage should be borne in the mind of the people involved in the rescue as they require, specialized techniques of handling the disaster. There can be possible collapse of buildings or other facilities, thus causing blockage of escape channels. The bomb explosions of Norfolk Hotel and American

Embassy in the Central District of Nairobi in 1992 and 1998, respectively caused deaths of more than 286 people, most of whom were trapped in the rubble of collapsed buildings (Mathenge et al, 1999). Kathangu (August, 1998) recounts that a group of 12 girls trapped in the rubble of Ufundi House, next to the bombed American Embassy, "had been talking to us through cracks of the rubble for more than three hours, but we could not be of any help due to lack of equipment and expertise". There is need to marshal resources to re-open the escape routes. There could be break up of fire and this will call for adequate fire extinguishing facilities. The institution may not have sufficient equipment for fire control and this may require seeking assistance from capable institutions.

This action has to be extremely expeditious because any delay may cause extensive damage and curtailment of further rescue measures. While assistance is being sought from other institutions, available fire-extinguishing equipments should be utilized to the maximum.

Chemicals may spill thus exacerbating the fire damage. In addition they will possibly bring about choking of the people within the vicinity and they may prevent rescue activities. In this case there may be need for vulnerable facilities to keep sufficient gas masks to ease evacuation in the event of spillage of chemicals. Switch-off facilities at strategic reticulation system need to have been put in place in order to switch off further spillage to the area that may have caught fire. Chemicals do not only choke but they can affect skin, nervous systems, under worst situation they may be radio active, thus affecting various organs of the victims. The spread is spontaneous and may cover many miles radius from the source. The Bhopal disaster of 1984, associated with leakage of methyl isocyanate into the air affected 200,000-300,000 people exposed to the contamination, thus causing death of more than 11,000 of them. Delayed response and limited knowledge of the toxic effects of methyl isocyanate accounted for significant number of deaths, which could have been otherwise avoided. Similar leakage of tetrachlorodibenzo-p-dioxin poisoning of Seveso, Italy caused 183 cases of human illness and death of 100,000 animals (French, 1999).

Death, injuries and shock will be inflicted on victims as a result of collapsed buildings, fire and chemical spillage. Dead bodies will be found in burned, mutilated or intact states. They will be found in open spaces, blocked corridors or buried under collapsed debris. There will be need for specialized evacuation expertise. Inexperienced rescuers may easily evacuate the intact bodies in open spaces, but mutilated or burned bodies may require the expertise of doctors and paramedical staff. The trapped bodies under debris may require combined expertise of architects, security and medical personnel.

After the US Embassy bomb explosion in Nairobi on 7th August 1998, 100 Israeli specialized soldiers with six sniffer dogs arrived in Nairobi on 8th August, 1998. They managed to retrieve many people alive and dead bodies from the rubble of Ufundi House next to the US Embassy building within 24hours. (Sunday Standard, August, 1998). Removal of the dead from such a disaster may be dangerous to the rescuers; special gloves, masks and vests may be necessary for the safety of the rescuers. Some rescues may be so massive that national capability may not cope with them. In this case there is a need to assess the situation in time and if it requires international intervention that should be done with all due speed.

Rescue of the injured people is equally delicate, and it requires specialized expertise. Injured people in open spaces are easier to deal with. However, trapped victims who can shout for help may take more than a day to rescue, and often some may die because of anxiety or choking despite tireless efforts of rescuers. Under such circumstances there is need for sociologists and psychiatrists intervention to reduce the anxiety of the victims. This may save lives of a significant number of the trapped victims. Victims who would have been otherwise saved lose their lives because of shock as a result of which they cannot shout for help. Such victims could only be saved by specialized technology whereby they are located by sensors.

It is not enough to remove victims, whether dead or alive from a chemical disaster. It is equally important to transfer the dead to mortuaries and the injured to hospitals. The latter category requires more speedy action to avoid further loss of lives. When military used poisonous gas against 4000 demonstrators in Georgia in 1989, 14 died and 707 of them were hospitalized, due to exposure of the gas. The main problem was that hospital staff could not identify the chemicals causing toxicity in time and thus many lives that could have been otherwise saved were lost (Katsistadze et al, 1999). Obviously, the institution that has been befallen by the calamity may not have sufficient transport to transfer the victims to mortuaries and hospitals. In this case there is need to mobilize any available transport including good samaritans, hospitals, rescue organizations, as well as security forces. Hospitals and mortuaries need to be notified in order to expect such an influx of victims and mobilize emergency resources to handle the situation.

Besides transportation facilities, there is need to mobilize resources for first aid on site including drugs, bandages, stretchers, blood transfusion etc. before live victims reach hospitals. Drugs and blood may not be available in hospitals. Consequently there is need to seek assistance of short notice from drug manufacturers and suppliers, and blood transfusion from nearby well wishers.

FOLLOW-UP

After most of the victims have been securely taken to hospitals and mortuaries there is immediate need to set up a Disaster Committee whose terms of reference would include, among others:

- Identification of victims and informing the next-of-kin about the same.

- Arrangements for burying the dead victims
- Counselling the injured victims and the relatives of all the victims
- Arrangements for any specialized treatment both locally, and abroad
- Insurance for the victims
- Investigation of the cause of the disaster and recommendations on how to avoid future similar ones
- Evaluation of the damage and the measures taken with a view to recommending future efficient action
- Transparent administration of the donations towards the disaster

It is worth noting that the number of the members of the committee need to be of diverse, but relevant specialized expertise, high integrity and long experience as the issues to be addressed are highly sensitive, not only at the national, but also international level.

The recommendations of the committee need to be carefully studied and integrated in any future policies and development programmes with a view to arresting chemical disasters before they occur as their adverse impacts on socio-economic development are enormous.

CONCLUSIONS AND RECOMMENDATIONS

Chemical disasters, as noted in the foregoing observations, will always occur despite the precautions taken. However this fact should not be a discouragement but rather a challenge that demands technological innovations for more advanced monitoring and deterrent measures.

Most lives are normally lost when a chemical disaster is accompanied by fire and collapsed buildings. The two make it difficult if not impossible for rescuers to save some lives, which could not have been otherwise lost as a result of delays in reaching the victims. In this regard there is need for any chemical establishment to have efficient fire fighting facilities and to mobilize specialized rescuers in the event of collapsed buildings. Such rescuers should be equipped with sniffing dogs and electronic sensors to identify sounds or movements of trapped victims under rubbles.

Many victims who arrive in hospitals alive have lost their lives because doctors are not immediately able to identify the chemicals affecting the victims. Data banks in hospitals for various chemical plants may help save many lives because the doctors may be able to tell the poisoning chemicals immediately, and thus administer antidotes without any delay.

Some of the worst chemical disasters are those which include leakages of chemicals which have long half life span in the water and in the air, and these may adversely affect communities who may be located very many kilometers away from the source of the disaster. Such a situation calls for wide and speedy dissemination of information and arresting the leakage expeditiously. Monitoring of the spread and effects of the chemicals should be intensified and remedial measures taken.

Chemical plants are the most vulnerable sites by terrorists or enemies during wars. In this regard surveillance and anti-missile facilities should be installed to reduce vulnerability.

Experience has shown that no single state can fully counter the adverse effects of chemical disaster. There is need for states, therefore, to ratify treaties, protocols or memoranda of understanding as instruments of cooperation in dealing with the disaster.

In any case every state should endeavour to train personnel in strategic institutions such as chemical plants, security and hospitals to handle such disasters even before mobilizing regional or international support. Sustainable public awareness should be created as this makes the operations of experts easier when a chemical disaster occurs.

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40. EPIDEMIOLOGY AND DETECTION METHODS OF THE MOST IMPORTANT INFECTIOUS DISEASES IN THE REPUBLIC OF MACEDONIA

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SUMMARY

The infectious diseases are still one of the most frequent and most important diseases. Because of their epidemiological characteristics, mortality and health damages, infectious diseases are very important social -medical problem.

During last three years period (97, 98, 99) the epidemiological situation in the Republic of Macedonia (2.2 million inhabitants) was classified as Insecure. The total number of infectious diseases was 74.475 (1997). 35.936 (1998), and 63.059 (1999). The frequency of the most important diseases, during 97,98,99 - respectively. were:

	97	98	99
Influenza:	53.199	20.451	39.403
Entero-collitis:	7088	9755	8727
Chickenpox:	5879	4986	5468
Hepatitis:	954	2047	3439
Toxinfection alimentary:	1813	2057	1822
Brucellosis:	785	538	459
Tuberculosis:	403	321	385
Dysentery:	228	390	134
Meningitis and Meningo-encephalitis:	162	184	178
Salmonellosis:	250	265	357
Anthrax:	9	3	3
HIV/Aids:	0	3	5
Tetanus:	1	1	4
Syphilis:	0	3	2

A total of 26 epidemics appear during viewed period, 9/9/8. Most important were Salmonellosis-12, Toxiinfectio alimentaris-4, Hepatitis-A- 5, Dysentery -2, Meningitis serosa-1, and Brucellosis-1. A total of 21 patients died, 6/ 8/ 7, mostly from Meningitis and Meningo-encephalitis-13, Tuberculosis – 6, and Tetanus-2.

During refugee's crisis in Macedonia, March-September-1999, (360.000 refugees), the epidemiological situation was under complete control. Increasing of infectious diseases in the country and within the refugees camps were not registered.

Besides routine-conventional diagnostic methods, sophisticated diagnostic methods, including ELISA, immune-fluorescence, and molecular diagnostic methods for some diseases, are in use.

Because the epidemiological situation in neighboring countries is the same or even worse, better collaboration in following-up and surveillance of epidemiological situation, promptly sharing of correct information (e-mail). experiences and advances in diagnostic, are necessary in preventing and control of spreading of the infectious diseases from one region to other.

INTRODUCTION

The infectious diseases are still one of the most frequent and most important diseases, although until recently. the long struggle for control seemed almost over (1). Because of their epidemiological characteristics, mortality and health damages, infectious diseases are very important social-medical problem. Infectious diseases are one of the leading causes of death (25% from a total of 53.9 millions, worldwide, 1998): 3,5 millions deaths from Acute respiratory infections, 2,3 millions from AIDS, 2,2 millions from Diarrhoeal diseases, 1,5 millions from Tuberculosis, 1,1 millions from Malaria, etc. (4). Infectious diseases are the main causes of death among children (63%), ages 0 to 4 years. Extreme poverty exposes over 1000 million people to the hazard of infectious diseases in their everyday lives. Up to half of all people on the earth are at the risk of many endemic diseases. The effects of climate change may allow some diseases to spread to new geographical areas. Microbes continue to evolve and adapt to their environment, adding anti-microbial resistance to their evolutionary pathways.

Continuing global population growth and rapid urbanization force many millions of city dwellers to live in overcrowded and unhygienic conditions, where lack of clean water and adequate sanitation are breeding grounds for infectious disease. Migration and the mass movement of many millions of refugees or displaced persons from one country to another as a result of wars, civil turmoil or natural disasters, also contribute to the spread of infectious diseases (1).

Infectious diseases range from those occurring in tropical areas (such as malaria and dengue hemorrhagic fever) to diseases found worldwide (such as hepatitis and sexually transmitted diseases, including HIV/AIDS). Transmission can occur by direct person-to-person contact, through insects and other vectors, by way of contaminated vehicles such as water or food, and in other more complex ways. Increasing international air travel, trade and tourism result in disease-producing organisms being transported rapidly from one continent to another.

Some cancers are caused by viruses, bacteria and parasites. WHO estimates that 15% of all new cancer cases could be avoided by preventing the infectious diseases associated with them. Cancer is the second most common cause of death in many parts of the world. Large reported outbreaks of known infectious diseases, 1998-1999 were: Cholera (45), Meningococcal disease (28), Enteric infections (20), Acute hemorrhagic fever (15), Acute respiratory infections (8), Anthrax (7), Yellow fever (6), Plaque (5), other (20), (2,3,4).

AIMS

To give an overview on the epidemiological situation of the most important infectious diseases in the Republic of Macedonia, in the last three years period, impact of refugee-crisis, and current detection methods of infectious diseases.

RESULTS AND DISCUSSION

Republic of Macedonia is a small country in the middle of the Balkan peninsula with about 2.2 million inhabitants and 25.713 km^2 territory. Besides Institutes of Microbiology and Epidemiology - Medical faculty Skopje and Veterinary faculty Skopje, there are twelve specialized institutions of preventive medical care:

I. Republic Institute of Health Protection – Skopje,

II. Regional Institutes of Health Protection: Skopje, Kumanovo, Stip, Tetovo, Strumica, Veles, Prilep, Bitola, Ohrid and Kocani,

III. Institute of Preventive Medical Care – Military Health Institution Centre – Skopje.

During last three years period (97, 98, 99) the epidemiological situation in the Republic of Macedonia (2.2 million inhabitants) was classified as Insecure. The total number of infectious diseases was 74.475 (1997), 35.936 (1998), and 63.059 (1999). The frequency of the most important diseases, during 97,98,99 - respectively, were: (5,6,7):

Influenza:	53.199	20.451	39.403
Entero-collitis :	7088	9755	8727
Chickenpox :	5879	4986	5468
Hepatitis :	954	2047	3439
Toxinfection alimentary :	1813	2057	1822
Brucellosis:	785	538	459
Tuberculosis:	403	321	385
Dysentery:	228	390	134
Meningitis and Meningo-encephalitis:	162	184	178
Salmonellosis:	250	265	357
Anthrax:	9	3	3
HIV/Aids:	0	3	5
Tetanus:	1	1	4
Syphilis:	0	3	2

A total of 26 epidemics appear during viewed period, 9/9/8. Most important were Salmonellosis-12, Toxiinfection alimentaris-4, Hepatitis-A- 5, Dysentery -2, Meningitis serosa-1, Brucellosis-1. A total of 21 patients died, 6/ 8/ 7, mostly from Meningitis and Meningo-encephalitis-13, Tuberculosis – 6, and Tetanus-2.

During refugee crisis in Macedonia, March-September-1999, over 360.000 refugees from Kosovo (17% of the population in Macedonia) entered into the country. Over 250.000 refugees were accommodated in eight camps, and over 100.000 in houses. Some days there were over 20.000 refugees coming into the country, and in some camps there were over 50.000. The institutions of preventive medical care that were in charge for epidemiological

surveillance and all epidemiological measures into the refugee camps were: Republic Institute of Health Protection – Skopje, Regional Institutes of Health Protection: Skopje, Kumanovo, and Tetovo, and Institute of Preventive Medical Care – Military Health Institution Centre – Skopje. All children up to three years got: Polio, Diphtheria - Tetanus-Pertusis, Measles, Rubella, Mumps and BCG vaccines. The epidemiological situation was under complete control. There were sporadic cases of Hepatitis-A, Diarrhoeal diseases, meningitis, Scarlet fever, but not any outbreak appear. There were no deaths from infectious diseases registered. There was no impact on significant increasing of number of infectious diseases in the country and within the refugee camps.

Beside routine conventional diagnostic methods, that are in use in all Institutions of preventive medical care, a sophisticated diagnostic methods including ELISA, immuno-fluorescence, and molecular diagnostic methods for some diseases, in some labs, are in use as well, but can't cover diagnosis of all, and some of emerging and re-emerging infectious diseases. In collaboration with Armed Forces Institute of Pathology, Washington DC, and Idaho Technology, USA, the RAPID PCR – (LightCycler PCR), lab will be established very soon in the Institute of Preventive Medical Care – Military Health Institution Centre – Skopje. The RAPID, as a fastest PCR (Polymerase Chain reaction), is a promising tool for rapid diagnosis and a proper response to infectious diseases and unexpected outbreaks, but collaboration with developed countries is necessary in set-up the tests, optimizing the reaction, supplying with specific reagents and sharing the experiences.

Because the epidemiological situation in neighboring countries is the same or even worse, better collaboration in following-up and surveillance of epidemiological situation, promptly sharing of correct information (e-mail), experiences and advances in diagnostic methods, are necessary in preventing and control of spreading of the infectious diseases from one region to other. The future of infectious disease control is likely to lie with vaccines rather than drugs but controlling infectious diseases is an imperative global challenge that requires a global response.

CONCLUSIONS

The epidemiological situation in reviewed period (1997-1999) was classified as Insecure, but without any impact on the epidemiological situation in the region. There were a total of 26 epidemics of infectious diseases with 21 deaths.

During the refugee-crisis, with over 360.000 refugees (17% of the population), the epidemiological situation was under control. Outbreaks and deaths from infectious diseases were not registered. There was no impact on significant increasing of number of infectious diseases in the country or within the refugee's camps.

There are very good diagnostic possibilities for the most of the infectious diseases, and promising advances for rapid detection and response introducing the molecular diagnostic methods. Controlling infectious diseases, which do not recognized any borders and limits in unexpected spreading, as an imperative global challenge for a global response, requires collaborations between all, developed, developing and undeveloped countries.

KEYWORDS epidemiology, infectious, diseases, outbreaks, detection, methods

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41. BIOMEDICAL SAMPLING: AN OPCW PERSPECTIVE

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The Chemical Weapons Convention provides for the collection of samples of biomedical origin during investigations of alleged use of chemical weapons by the OPCW (Verification Annex, Part XI, paragraphs 16 and 17). Analysis of these samples may provide information that contributes to an inspection team's conclusions regarding allegations of the use of chemical weapons.

While the Preparatory Commission for the OPCW, and the OPCW itself have both devoted considerable resources to the development of procedures and a network of accredited laboratories for the collection, transport and analysis of environmental samples, similar arrangements are not yet in place for samples of biomedical origin. Although some of the principles developed for environmental samples may also apply to samples of biomedical origin, there are also some distinct differences. In many Member States, different laboratories would be involved, and there is currently no international agreement on the most suitable techniques to use for the analytical process.

A meeting of international experts in this field took place at the OPCW Headquarters in The Hague on the 6th and 7th of December 1999. The experts were invited to review the current status of international abilities in this area, and to develop recommendations for addressing current deficiencies.

This presentation reviews the conclusions and recommendations of the expert group, and is based on the report of that group.

The group recognized that there is currently a gap in the international community's ability to acquire and analyze biomedical samples according to the provisions of the Chemical Weapons Convention for investigations in cases of alleged use of chemical weapons. While a network of designated laboratories is in place, their current scope of accreditation does not include the analysis of biomedical samples. As no proficiency testing for analysis of biomedical samples has yet occurred, a demonstrated capability does not exist. However, the group felt that the bulk of the principles already agreed for the handling of environmental samples could be applied to creating a system for biomedical sampling and analysis.

ON-SITE SAMPLING AND ANALYSIS OF BIOMEDICAL SAMPLES

In general, the OPCW draft procedures for acquiring and transport of samples seem adequate for present analytical methods. However they need to be validated in conjunction with inter-laboratory comparison exercises and proficiency tests for analytical techniques. Sample size and procedures for preservation must be consistent with the analytical methods used. Procedures should be approved for the packaging and transportation of biomedical samples.

The functions and principles currently in place for the handling of environmental samples during an OPCW inspection should be applied to biomedical samples. Although on-site analysis is the preferred approach, there is currently a lack of analytical procedures which can reliably be used for biomedical sample analysis on-site. The OPCW's approach to biomedical sample analysis should allow for the inclusion of on-site analysis as suitable technology becomes available. At present, screening of butyrylcholinesterase and acetylcholinesterase activity for enzyme inhibitor exposure can be used on-site to guide further actions. In addition, immunoassays for some toxins are available for use on-site.

Splitting of samples (authentic and background) to be transferred for off-site analysis should only be performed on-site. On-site sealed authentic sample aliquots should not be opened, unsealed or divided before their arrival at a designated laboratory. This implies that the inspection team should be capable of performing all sample processing activities, to include, for example, plasma separation, cooling or freezing, and anticoagulation.

Wherever possible, background samples should be requested from unexposed local individuals forming an appropriate control group as uniform as possible. Part of the background sample could be spiked at the OPCW laboratory with appropriate target analytes to serve as positive controls.

The concepts of sample splitting, control samples, and spiked samples are relevant also for biomedical samples. Non-uniform distribution of the agents or their metabolites in tissue samples, and availability of materials and procedures for spiking biomedical samples may pose a problem.

Procedures which minimize the biohazard associated with biomedical samples are important for safe handling.

THE ROLE OF THE OPCW LABORATORY

The group recommended that the OPCW laboratory should play a similar role for the handling and storage of biomedical samples as is currently intended for environmental samples. The major difference in terms of preparation for this role is likely to be with regard to procedure development and training, as opposed to the need for additional facilities and equipment. These requirements should be further elucidated by the working group.

THE ROLE OF DESIGNATED LABORATORIES

The group further recommended that the currently approved OPCW decisions and working system for designated laboratories be directly applied to the handling of biomedical samples. The principles previously used for accreditation of sampling and analytical procedures of environmental samples are directly applicable to the process of extending the scope of accreditation of currently designated laboratories to the analysis of biomedical samples, and for designation of new laboratories for that purpose. Laboratories seeking designation by the Director General of the OPCW for the analysis of biomedical samples should obtain accreditation by an internationally recognized accreditation body for that purpose.

As an important first step, inter-laboratory comparison exercises will need to be conducted with scenarios focused on specific categories of scheduled chemicals and/or their metabolites (including adducts). Following the conduct of inter-laboratory comparison exercises, and taking into account the lessons learned from those exercises, proficiency testing should be started for the designation of laboratories for the analysis of biomedical samples.

All accredited or validated analytical methods should be acceptable to the OPCW provided they contribute to meeting the criteria for unequivocal identification. These criteria need further development, as some of the existing criteria for environmental samples may not always be applicable. This applies to different analytical methods for the same agent, metabolites (including adducts) or to different metabolites (again including adducts) derived from the same agent.

At present the following procedures have been published in open literature:

Nerve agents:
Measurement of butyrylcholinesterase activity in plasma
Measurement of acetylcholinesterase activity of erythrocytes based on hemoglobin content
Analysis of unchanged nerve agent in blood samples
Analysis of low molecular weight metabolites in blood and urine
Analysis of phosphylated proteins in blood and tissue

Mustard:
Analysis of unchanged agent in blood and tissues
Analysis of DNA and protein adducts in blood and tissues
Analysis of DNA and protein adducts in the skin by immunoassay
Analysis of unequivocal metabolites in urine

Lewisite:
Analysis of hydrolysis products in blood and urine
Analysis of adducts in blood

Other agents:
Analytical procedures for some toxic industrial chemicals are available.

Details of these procedures will be presented in a separate presentation during this Symposium. Applicability of these procedures for use in OPCW context still needs to be evaluated. This will identify gaps in knowledge where further development can be encouraged (for example, riot control agents).

In general, quantitative methods are superior to qualitative methods and are desirable for testing of biomedical samples wherever possible. However, as with environmental samples, a qualitative identification may be acceptable. Analytical methods should address minimal detectable concentrations. Inter-laboratory comparison exercises and proficiency testing should demonstrate that the laboratory can detect concentrations in spiked samples that are relevant to the OPCW inspection activities.

CONCLUSIONS

The work of the group clearly indicated that while there is much that could be applied from experience gained from developing a sampling and analysis system for environmental samples, much remains to be done before the international community will have at its disposal a comparable system for biomedical samples. A co-operative and facilitative approach by experts and laboratories of OPCW Member States will be required to address this gap. Decisions by the OPCW's Conference of States Parties on priority, budget, and program methodology will be an important first step for progress in this regard.

KEYWORDS

Biomedical sampling, analytical techniques, chemical weapons, retrospective diagnosis

42. THE INTERFERENCE OF STRESS ON PHYSOSTIGMINE PRETREATMENT AGAINST SOMAN INTOXICATION IN GUINEA PIGS

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INTRODUCTION

There is growing evidence that stress occurring during military operations can impair the efficacy and appearance of side effects of medical treatment. Normally, research efforts toward finding an effective treatment against intoxication with organophosphorus (OP) and acetylcholinesterase (AChE) inhibitors are performed in a standard laboratory situation. However, in a more realistic situation other factors may interfere with the treatment regime. After the Persian Gulf war soldiers were suffering from symptoms called the Gulf War Syndrome. Most symptoms were hard to determine which could be due to interaction with other drugs. Some symptoms could be the result of the stress situation in wartime. It is known that stress can change the kinetics of the pretreatment (1) and, therefore, affect the protective ratio and evoke the appearance of side effects. During operation Desert Storm soldiers were given 30-mg pyridostigmine (PYR) tablets (one every 8 hours). This dose of PYR was expected not to show undesirable cholinergic effects. Nevertheless, peripheral and central side effects were recorded (2). The central effects could be the result of stress. First of all stress itself could be an important factor. Stress induces a prolonged stress-induced corticosterone secretion that leads to a reduction of hippocampal corticosteroid receptors. This decrease of receptors influences other transmitter systems, like acetylcholine (ACh), in the hippocampus. Indeed, the hippocampal ACh release seems to be increased after stress (3). Furthermore, stress also enhances the passage across the blood-brain barrier (1). In operation Desert Storm nine cases of pyridostigmine self-poisoning were encountered. These individuals only suffered from peripheral cholinergic symptoms such as abdominal cramps, diarrhea, hypersalivation, blurred vision etc, whereas no effects on the central nervous system (CNS) were observed (4). This finding strengthens the idea that a combination of other factors, like stress during the operation, plays a role in the appearance of side effects. Therefore, the protective efficacy and the side effects of the pretreatment should also be examined in stressful situations. Such circumstances may evoke the appearance of or strengthen unwanted side effects and decrease protection against intoxication. Pretreatment with physostigmine (PHY) has proved to be very effective against sarin or soman-intoxication (5). Furthermore, PHY protects more effectively against soman intoxication than PYR in rats (6,7) and in guinea pigs (8) under standard test conditions. In this study the effects of stress on side effects of PHY (0.025 mg/kg/hr) pretreatment and its efficacy against soman intoxication were determined in guinea pigs. To prevent unwanted side effects due to AChE inhibition in the central nerve system (CNS) by PHY, the pretreatment was combined with the muscarinic receptor antagonist scopolamine (SCO) (0.018 mg/kg/hr) (9). Stress factors were chosen to represent military conditions: emotional stress, physical stress and psychological stress. Most effects can be expected to be centrally mediated effects that may induce changes in different types of behavior. For this reason behavioral read-out systems were used to elucidate the severity of PHY side effects and soman induced incapacitation.

MATERIALS AND METHODS

Animals

Male Dunkin-Hartley albino guinea pigs CrL: (HA)BR (Charles River) with an initial body weight of 400-450 g were used. The animals were kept singly in a cage (Makrolon type IV). The ambient temperature was regulated between 20-22°C. Relative humidity was monitored but not regulated and was kept over 50%. Food and water were always available. The experiments described here received prior approval by an independent ethical committee.

Drug solutions and implantation of osmotic mini-pumps

Physostigmine (eserine) and scopolamine bromide were obtained from Sigma (St. Louis, U.S.A.). Atropine Sulphate was obtained from ACF (Amsterdam, The Netherlands). Soman (O-pinacolyl methylphosphonofluoridate) was synthesized at the Prins Maurits Laboratory TNO. Alzet® Osmotic Mini-pumps with a constant delivery rate of 0.55 µl/hr (Model 2002, Alza Corp., Palo Alto, USA) were used to deliver either

the vehicle or the combination of PHY (0.025 mg/kg/hr) and SCO (0.018 mg/kg/hr). This dose of PHY offers the recommended blood-AChE inhibition of about 35 % (8). SCO (0.018 mg/kg/hr for a period of ten days) leads to a SCO plasma concentration of 45 nM (8). This was comparable to the level found after a single SC injection of 0.1 mg/kg SCO (43 nM). This SCO plasma concentration did not lead to behavioral side effects and could antagonize PHY induced side effects (9, 10). The vehicle consisted of 20% propylene glycol, 10% ethanol and 70% water (1 part glacial acetic acid in 2000 parts distilled water). The drugs used were solved in the vehicle. Because the animals gain weight during the pretreatment period, the PHY and SCO concentrations were based on the estimated weight of the animals one week after implantation based on the normal growth curve for guinea pigs in our laboratory. The pumps were implanted subcutaneously on the backs of the animals under ketamine/ventracil anesthesia. The wounds were sutured with woundclips.

Study design

The current study was performed in two different treatment groups of animals (n= 8 animals/group). Both groups were pretreated with PHY (0.025 mg/kg/hr) and SCO (0.018 mg/kg/hr) during 11 days, intoxicated with 2x LD_{50} soman intoxication at day 11, and after one minute followed by a post intoxication therapy with atropine sulphate (AS)(0.36 mg/kg im). The LD_{50} dose of soman (applied subcutaneously) used was 24.5 μg/kg (11). The animals of one group (non-stress group) were handled by the standard procedures and the animals of the other group (stress group) were exposed to eight weeks of intermittent variable, unpredictable and uncontrollable stress. The stress factors consisted of cold stress (30 min in a refrigerator), psychological stress (foot shocks with an interval of 10 min), physical stress (swimming task and running wheel), emotional stress (placing the animal in an unfamiliar territory for 30 min).

After the animals were trained in a conditioned learning task, the shuttlebox, the baseline values of the different read-out systems were collected. The body weight, plasma cortisol level, blood-AChE activity (for testing the efficacy of the osmotic pumps), shuttlebox, startle response, and exploration activity in the Open Field (OF) task were determined and registered. Subsequently, based on the results obtained, two matched subgroups of 8 animals each were formed that showed no significant differences in any of the behavioral tests. Thereafter, the animals from the stress group received the daily stress factors (with exception of the weekends). Once a week the shuttlebox performance, the startle response, and the body weight were measured/registered. Every other week the animals were tested in the OF task and blood samples were collected for measuring the plasma cortisol level. After six weeks of stress induction, Alzet® osmotic mini-pumps, containing PHY and SCO, were implanted in all animals. This was called day 0. During the pretreatment period the animals from the stress group were still exposed to the daily stress occasions. At day eleven of the continuously administered pretreatment all animals were intoxicated with 2x LD_{50} soman. One minute after the soman treatment the animals received a post-intoxication therapy with AS. The osmotic pumps were not removed.

The efficacy of the PHY and SCO pretreatment with or without stress treatment in counteracting soman-induced post intoxication incapacitation was investigated by observing the post intoxication symptoms, like hyper-salivation, tremors and convulsions immediately after soman intoxication, and by measuring behavioral parameters after the intoxication symptoms became less severe. These tests started 2 hours after soman intoxication (day 11) and were repeated at day 12, 13, 14 and 18.

Behavioral tests

1) Shuttlebox performance: In this test the active avoidance of an unpleasant event upon a conditioned stimulus (CS) is used to measure the retrieval of learned behavior. For this test an automated two-way shuttlebox, consisting of two equal compartments of 23x23x23 cm with rounded corners, connected by a photo-cell-guarded gate, is used. The animals have to learn how to avoid a stream of air (about 6 l/s, air tube diameter 1 cm) aimed at their fur within 10 s after presentation of a sound stimulus, the CS. During the daily training and test sessions the animals receive 20 trials at an intertrial interval of 20-30 s (random). Only animals that reach the criterion of 80% or more correct avoidance reactions (CARs) after training, were used in the experiments (10). The number of CARs was used to express the active avoidance performance.

2) Open Field task: This technique is used to measure parameters of spontaneous behavior, like locomotor activity and exploration in a quantitative way (12).

The OF consists of a black field of 100 x 100 cm, with 25 cm high enclosing walls. A black grating covers the top of this box. The test room is homogeneously illuminated (100 lux) with a background noise of 52 dB. A

video camera is placed above the OF for registering the movement patterns of the white animal in the black area during a 10 min session. The moving patterns are downloaded into a computer. The following parameters were studied: 1) the distance run, 2) the time spent in the inner field, i.e. a 60x60 cm virtual area in the center of the field (The remaining part of the field is defined as outer field,), 3) the number of crossings from outer to inner field, and 4) the number of times the rat changes corners. Corners are defined as virtual squares of 20x20 cm in each corner of the field. All parameters are expressed in a cumulative fashion. 3) Auditory startle response: In this test the stretching movement of the hind paws is used to reflect the reaction of the animal on a startle signal (13). For this test the animals are exposed to 20 auditory startle pulses (120 dB, 10 kHz, 20 ms) while standing with their hind paws on a platform in a vertically mounted PVC-tube (diameter 7 cm, length 16.5 cm). The startle response of 200 ms duration is measured by a transducer connected with the platform, registering the force exerted by the animal upon presentation of the stimulus. An AD converter of an IBM-compatible PC digitized the responses. The area under the curve (AUC), amplitude and latency of the startle response are registered and used to express the motor reaction of the startle reflex.

Determination of cortisol plasma levels

Cortisol plasma levels were determined using a cortisol-kit of ICN. Blood (about 60 μl) obtained from the ear vein of the guinea pig were mixed with heparin and centrifuged for 8 min at 2000 g. The supernatants were stored at -20°C. Within 10 days the cortisol plasma level was determined. 25μl Plasma was applied in antibodies coated tubes. Thereafter 0.5 ml of a solution with 125 I-cortisol was applied. This radioactive cortisol binds in competition with the plasma cortisol. Thereafter bound and unbound radioactivity was separated and bound radioactivity was counted.

Statistics

For statistical analysis of the behavioral tests an analysis of variance (two-way ANOVA) was used. For the symptomatology after soman intoxication a Fisher exact probability test or an unpaired t-test with Welch's correction was used. In all tests p values < 0.05 were considered significant.

RESULTS

In this study the effect of stress alone, on the appearance of side effects during PHY and SCO pretreatment, and on the efficacy of the pretreatment in preventing the toxic influences of 2x LD_{50} soman was tested.

Effect of the stress procedure alone

Plasma cortisol levels were measured every two weeks. Blood samples were collected before stress induction, 15 min and 60 min after stress induction (see Fig. 1). The intermittent variable, unpredictable and uncontrollable stress used in this study, induced a strong increase of the plasma cortisol levels measured after 15 and 60 min after the stress induction (at both time-points $p < 0.05$). There was no difference found between 15 and 60 min after stress induction on the increase of plasma cortisol.

During the first three weeks of intermittent variable, unpredictable and uncontrollable stress all animals from the stress group showed a significantly higher number of intertrial response (ITR) in the shuttlebox (ITR non-stress group: 2.21 ± 0.34, ITR stress group: 7.63 ± 0.88; $p < 0.05$). However, after the first three weeks of stress induction the stressed animals reacted similarly to the non-stress animals in the shuttle box. The activity in the OF task, on the other hand, was not affected (see also Fig 2). On the startle response a tendency towards an increase of the startle response (amplitude and AUC) was found (see also Fig 3). This effect was not found to be significant.

Effect of stress on side effects of PHY and SCO pretreatment

During the 11 days of continuously applied pretreatment of PHY and SCO no effect was found in all test systems used under the standard conditions. Under the stressful conditions, on the other hand, an increase was found in the OF task: the entries corners and inner field were significantly increased (Fig 2; p=0.028 and p=0.022 resp.).

Effect of stress on the efficacy of PHY and SCO pretreatment against 2x LD_{50} soman

Protection against soman induced lethality

All animals of both groups (stress and non-stress) survived the 24 and 48 h criteria after intoxication with 2x LD_{50} soman. Four days after soman (day 15) one animal of the non-stress group died and one day later (day 16) one animal from the stress group died.

Post-intoxication incapacitation symptoms

The post-intoxication symptoms observed following soman intoxication are summarized in Table 1. The appearance of symptoms is significantly different between stress versus non-stress (two-way ANOVA, $p = 0.004$)

Behavioral post-intoxication incapacitation

All animals of both groups were able to perform the task in the shuttlebox; they showed a normal ITR activity (compartment changes during the inter-trial interval) after soman intoxication. Their performance was significantly decreased from 96.9 ± 1.3 to 28.8 ± 8.8 in the stress group and from 93.8 ± 2.3 to 38.8 ± 8.9 in the non-stress group. No significant difference was found between the two test groups. 24 Hours later this effect was slightly improved, but the effect was still present one week after soman. The effects on the startle response observed after 2x LD_{50} soman intoxication are shown in figure 3. In both test groups an increase of the startle response was observed. This effect was more persistent in the stress group.

DISCUSSION

In this study the effects of exposure to variable, unpredictable and uncontrollable stress on PHY and SCO pretreatment in a therapeutically relevant dose (14), against 2x LD_{50} soman was tested. This was done by comparing two test groups: stress versus non-stress. All other factors were kept equal. Three aspects were studied: the effects of the stress procedure on the test systems used, the appearance of side effects during pretreatment, and the protection against post-intoxication incapacitation after intoxication by 2x LD_{50} soman.

The stress procedure only affected the behavior in the shuttle box during the first three weeks (six weeks in total). The animals showed an increased inter-trial response (ITR: compartment changes during the inter-trial interval). This could be explained as a higher activity of the stressed animals. However, in the OF task no increase of the distance run (a measurement of activity) was observed. Presumably the increase of ITR was due to increased alertness. In case the effect on learning was tested, these animals would learn faster than the non-stressed animals. This is in accordance with the results obtained by Douma et al. (15). They blocked the mineralo-corticoid receptor which displays a high concentration and distinct distribution in the hippocampus, a brain region which is directly involved in the regulation of spatial orientation and learning. This blockade impairs cognitive behavior. However, in our experiment only the effects on learned behavior (memory) was tested. Both groups performed at their maximum level. This alertness effect was also found on the startle response: a rather insignificant increase of the startle reaction was found during the six weeks of stress induction which had disappeared after starting with the PHY and SCO pretreatment.

During the PHY and SCO pretreatment period of 11 days an effect was found in the OF task. The performances in the other test systems were not affected. In a previous study we tested the side effects of PHY and SCO following the same procedures as in the non-stress group. No side effects were observed in the shuttle box, startle response and neurophysiological parameters (9). In former studies we had never tested guinea pigs in the OF task. It was shown that the pretreatment with PHY and SCO also did not affect this task (Fig. 2). Remarkably, the OF seems to be the only task in which side effects were found in the animals of the stress group. The type of effects (increase of "entries corners" and "inner field") corresponded with an increased activity. This activity which was also found (although not to be significant) in the "distance run" parameter is presumably due to stimulation of cholinergic receptors induced by the increase of ACh induced by AChE-inhibition after PHY and due to the increased release after stress (3).

The high protection which was found in this study is in accordance with our previous study. The addition of SCO to the pretreatment or addition of AS as post intoxication therapy enhanced the protection synergistically against soman induced lethality (16). Only one animal of the non-stress group died after four days. This animal did not show the worst symptoms after soman (12.9 % convulsions observations of the total scoring hits: the mean value was 28.8 %). Presumably there were other factors that played a role in the condition of that animal. It acted quite differently in the behavior tasks before any treatment: there was a very high "time spent in inner field" in the OF task, a very small reaction on the startle reflex and a very gradual and late training curve in the shuttlebox performance. The animal died after a period of diarrhea. In the stress group also one animal died after a

period of dyspnoea five days after soman. This animal exhibited the worst post-intoxication symptoms. If no AS post intoxication therapy or no SCO was added to the pretreatment, the protection in a standard laboratory situation was not 100% (16). In case only PHY was added to the pretreatment all animals died within 24 hours. Therefore, we have chosen the complete treatment (PHY, SCO and AS). It could be that the scenario without SCO or AS would show a bigger difference on lethality between stress and non-stress.

That there is a difference between stress and non-stress animals can be concluded from the observations of the post-intoxication symptomatology. All stressed animals showed severe tremors and five of them had convulsions as opposed to 3 in the non-stress group. Dyspnoea was only found in the stressed animals. In a former study effects like dyspnoea were found only in animals without a post-intoxication therapy with AS (16). Therefore, these effects in a stressful situation could be the results of a combination of an increase of ACh release and AChE-inhibition or the system become more susceptible to ACh. Indeed after inescapable stress the ACh release was significant increased in the hippocampus and prefrontal cortex investigated with microdialysis technique (3), and the maximal number of muscarinic receptors (Bmax) in several brain areas such as the cortical layers, the CA1 field of the hippocampus and caudate-putamen was significantly increased (17). Furthermore, it could be that the blood-brain barrier permeability was increased through which PHY and soman could more easily enter the brain. It seems that already short-lasting immobilization stress shows this effect (18). Even when it is corrected for the decreased cerebral blood flow, a higher penetration into the CNS was found (19).

The animals from the non-stress group showed the best protection against the soman induced intoxication incapacitation. After soman a high increase of the startle amplitude and AUC were found in both groups. In the stress group this effect is more persistent: an increase or a tendency towards an increase of the startle response was still found after one week.

In a previous study it was clarified that direct effects on nicotinergic receptors were involved in the effects on the startle amplitude instead of AChE inhibition (20). It is known that soman has besides its AChE-inhibiting effect, like PHY (21, 22), direct effects on nicotinergic receptors (23). Furthermore, effects on the release of 5-HT after stress may play a role on the startle reflex (13). Serotonin also seems to play a role in the increased permeability of the blood-brain barrier under stress conditions (24).

From the present experiments it can be concluded that stress indeed influence the efficacy of the pretreatment. This can be due to the changes in the blood-brain barrier and other cholinergic effects, like the increased release of ACh and up-regulation of muscarinic receptors. Although PHY already easily penetrate into the brain because of its structure, stress also influences the appearance of unwanted side effects of the pretreatment. This effect would be worse in case a drug is used that normally hardly enters the brain, especially when this drug is only tested in a standard laboratory situation.

In conclusion, in a more realistic situation stress seems to interfere with the pretreatment against soman intoxication. Stress evokes the appearance of side effects and decreases protection against soman intoxication. Therefore, other risk factors should be incorporated in the experimentation set-up and not be ruled out during research in the area of treatment by medication.

ABSTRACT

The efficacy of a treatment against nerve gas exposure is normally tested in a situation in which the conditions are kept standard. During military operations no standard circumstances are available. Soldiers are exposed to mixtures of chemicals and to physical, emotional and psychological stress factors. All these factors may play a role in the efficacy of any treatment. Therefore, the side effects and efficacy of physostigmine as a pretreatment against 2x LD_{50} soman intoxication were studied in guinea pigs under intermittent variable, unpredictable and uncontrollable stress conditions. The exposure to stress factors during PHY-pretreatment (0.025 mg/kg/hr) leads to an increase of motor activity and after 2x LD_{50} soman exposure in pretreated animals to more sever intoxication symptoms, and more persistent incapacitation effect on the startle response.

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KEYWORDS
Behavior, guinea pigs, physostigmine pretreatment, soman intoxication, stress

NOTES
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FIGURES AND TABLES

Figure 1: Plasma cortisol levels (in nmol/l) measured in stressed guinea pigs and in non-stressed guinea pigs after 2, 4, 6, and 8 weeks of stress induction. During week 7 and 8 all animals were also pretreated with PHY and SCO. The cortisol values of the stressed animals were measured before (stress c), 15 min, and 60 min after stress induction. All values after stress induction were significantly increased (ANOVA and Newman-Keuls post-hoc test, $p < 0.05$).

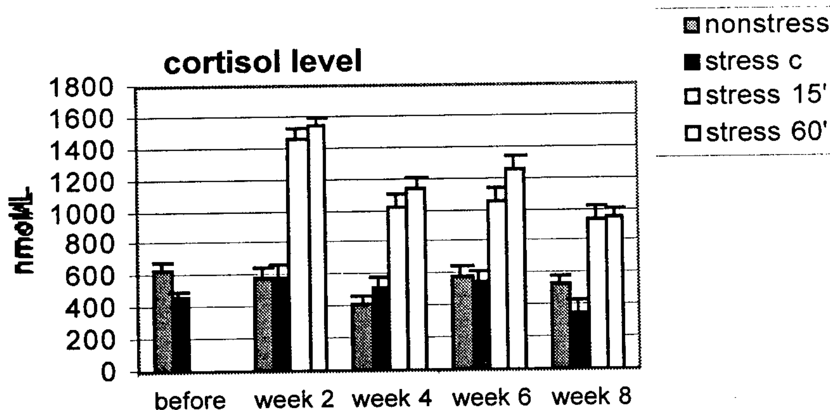

Figure 2: Performance in the OF measured in two different groups (n=8/group), non-stress or stress, before stress induction, after 6 weeks of stress induction (stress group), after 10 days of PHY and SCO pretreatment, two hours and 1 week after 2xLD50 soman. The distance run was expressed as the cumulative meters walked during the 10 min session (mean ± SEM). The entries corners or inner field were expressed as the cumulative number of entries in these virtual areas during the 10 min session (mean ± SEM). * Significantly different from non-stress.

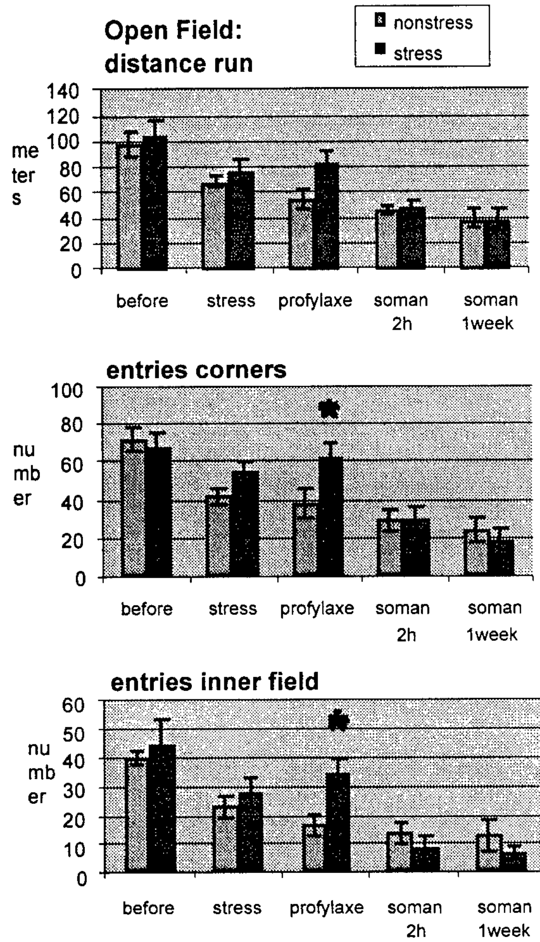

Figure 3: Area of the curve of the startle response of 200 ms duration (startle pulse: 20 ms, 120 dB, 10 kHz). Registration of the effects before, after 6 weeks of stress induction, after 10 days of PHY/SCO pretreatment and after soman intoxication (2, 24, 48 h and 1 week) in non stressed and stress guinea pigs (n=8/group, mean ± SEM).

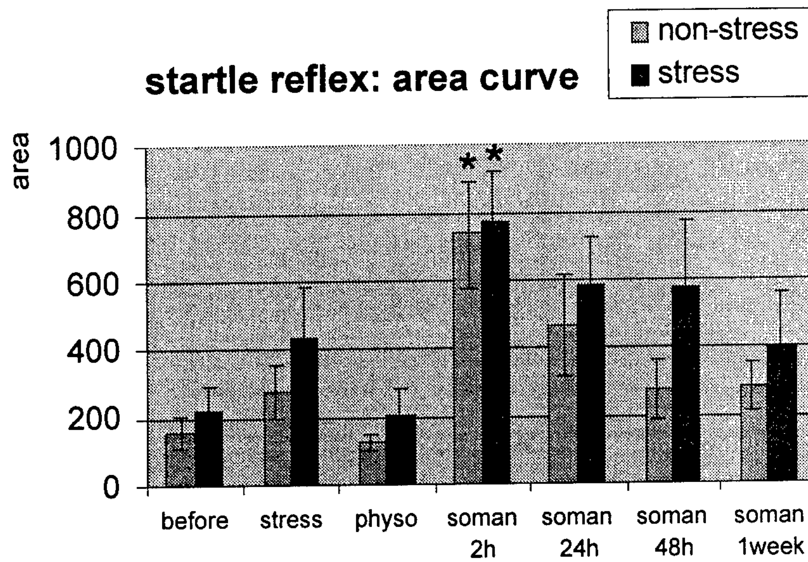

Table 1: Post-intoxication symptomatology after 2x LD_{50} soman and AS in continuously PHY and SCO pretreated guinea pigs under standard conditions (non-stressed) or stress-full conditions (stressed) expressed as the % of the total scoring.

non-stress symptoms	animal number 5	9	10	12	13	14	20	21	total animal	severity: % of scorings
chewing	8.2	20.0	6.9	20.7	12.9	7.5	0	0	6/8	12.7 ± 2.6
hyper-salivation	0	0	0	0	0	0	0	0	0/8	
mild tremors	25	54.3	72.4	10.3	54.8	38.5	23.1	54.5	8/8	41.6 ± 17.4
severe tremors	0	28.7	17.2	24.1	38.7	30.8	0	13.6	6/8	25.5 ± 3.8
convulsions	0	0	0	62.1	12.9	11.5	0	0	3/8	28.8 ± 16.6
dyspnoea	0	0	0	0	0	0	0	0	0/8	
total symptoms	2	3	3	4	4	4	1	2		

stress symptoms	animal number 3	6	8	15	16	17	19	22	total animal	Severity: % of scorings
chewing	12.2	57.9	69.7	53.6	31.4	51.9	28.6	63.6	8/8	16.3 ± 4.0
hyper-salivation	0	0	0	0	14.3	0	17.9	0	2/8	16.1 ± 1.8
mild tremors	65.0	52.6	48.5	42.8	22.9	42.9	21.4	50.0	8/8	43.3 ± 5.2
severe tremors	40.8	34.4	18.2	35.7	31.4	29.5	17.9	31.8	8/8	30.0 ± 2.9
convulsions	30.6	0	0	13.9	37.1	11.1	35.7	0	5/8	25.7 ± 5.5
dyspnoea	4.1	0	0	0	40.0	0	3.6	0	3/8	15.9 ± 12.1
total symptoms	5	3	3	4	6	4	6	3		

Total animal: number of animals in which the symptom was observed.
Total symptoms: total number of different symptoms observed in the animal. Severity of the symptom expressed as the % of total scoring hits of the animals in which the symptom was observed.

43. METHODS FOR RETROSPECTIVE DETECTION OF EXPOSURE TO TOXIC SCHEDULED CHEMICALS: AN OVERVIEW

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INTRODUCTION

Methods to analyze toxic scheduled chemicals and their decomposition products in environmental samples have been developed and are used, *e.g.*, to test the proficiency of laboratories to act as so-called designated laboratories for the OPCW. However, methods for such analyses in biological samples have only recently been developed. while the use or alleged use of chemical warfare (CW) agents in war and terrorism has clearly established an urgent need for biological markers of poisoning, *e.g.*, in blood and urine samples.

Retrospective detection of exposure to toxic scheduled chemicals can be useful for various reasons. First of all. such analytical methods can be used to establish firmly whether casualties have indeed been exposed to these chemicals. whereas dosimetry of the exposure will be a starting point for medical treatment of the intoxication. Second. these methods will be useful for verification of alleged non-adherence to the Chemical Weapons Convention. Especially in this application it appears that maximal retrospectively, preferably over a period of several months. is essential. Moreover, these methods can be used in a variety of other applications, *e.g.*, for health surveillance of workers in destruction facilities of CW agents and in forensic analyses in case of suspected terrorist activities.

In this report an overview is presented of the methods currently available for detection of exposure to sulfur mustard, nerve agents, lewisite and phosgene.

SULFUR MUSTARD

Sulfur mustard is a strong alkylating agent that reacts readily with nucleophiles under physiological conditions. The reaction products of sulfur mustard with these nucleophiles are all potential biological markers of human poisoning. Metabolites derived from an initial reaction with water and glutathione are excreted in urine (1-4). Adducts to DNA which may be present in various tissues and blood can conveniently be detected by using an immunochemical assay (5). In this section we will focus on adducts to hemoglobin and albumin, since it is expected that they are persistent and will allow retrospective detection.

Upon incubation of human blood with sulfur mustard, it appears that 20-25% of the dose was covalently bound to hemoglobin (6). The most abundant adduct was the histidine adduct (7). In addition. the adducts to cysteine. glutamic and aspartic acid and to the N-terminal valine residues were detected (7,8). As a biological marker of poisoning, N-alkylated N-terminal valine has the advantage that it can be selectively cleaved from hemoglobin by a modified Edman procedure using pentafluorophenyl isothiocyanate as reagent (9). Analysis of the resultant pentafluorophenyl thiohydantoin, using negative ion GC-MS-MS after further derivatisation with heptafluorobutyric anhydride, provided a very sensitive method for the detection of the N-alkylated valine (10). *In vitro* exposure of human blood to ≥ 0.1 μM sulfur mustard and *in vivo* exposure of guinea pigs could be detected employing this method. Moreover, the adduct could be detected in samples from victims of accidental exposure to sulfur mustard and CW casualties (11,12). Recently, a standard operating procedure (SOP) for determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin was developed. By using this SOP, it was found that the N-terminal valine adduct in globin of hairless guinea pigs and marmosets subsequent to I.V. administration of sulfur mustard (0.5 LD50) is persistent for at least 56 and 94 days, respectively. The SOP could be properly set up and carried out at another institute (U.S. Army Medical Research Institute of Chemical Defense, Edgewood, MD, USA) within one working day.

Recently, sulfur mustard has been shown to alkylate a cysteine residue in human serum albumin (13). The site of alkylation was identified in a tryptic digest of albumin from blood exposed to [^{14}C]sulfur mustard. A sensitive method for its analysis was developed based on Pronase digestion of alkylated albumin to the tripeptide S-[2-[(hydroxyethyl)thio]ethyl-Cys-Pro-Phe, and detection using micro-LC-MS-MS. *In vitro* exposure of human blood to ≥ 10 nM sulfur mustard could be detected employing this method. The analytical procedure was successfully applied to albumin samples from Iranian casualties of the Iraq-Iran war.

NERVE AGENTS

Five approaches have been explored in order to detect exposure to a nerve agent:

(i) measurement of acetylcholinesterase (AChE) inhibition (14,15),

(ii) detection of the intact agent,

(iii) detection of hydrolysis product of nerve agents,

(iv) analysis of phosphylated butyrylcholinesterase (BuChE) and, more recently,

(v) determination of phosphylated serum albumin (16).

In this paper we will focus on items (iii) and (iv).

Hydrolysis products of nerve agents

Metabolism of phosphonofluoridates and V agents in mammals leads predominantly to hydrolysis products, *i.e.*, the corresponding O-alkyl methylphosphonic acid and a minor amount of methylphosphonic acid. Methods for analysis of these compounds are based on GC-MS (*e.g.*, 17-19) or on LC-MS (*e.g.*, 20-22). For instance, the hydrolysis product O-ethyl methylphosphonic acid has been determined by GC-MS, after derivatization, in serum collected from a victim poisoned by VX (23). Recently, a convenient and rapid LC tandem MS method was developed for quantitative determination of O-isopropyl methylphosphonic acid (IMPA), *i.e.*, the hydrolysis product of sarin, in blood and urine (24). Sample work-up was limited to a single extraction step. This method could be successfully applied to the analysis of serum samples from the victims of the Tokyo subway attack by the Aum Shinrykio sect and from an earlier incident at Matsumoto. It is envisaged that this method can be used for analysis of other hydrolyzed nerve agents as well.

Advantageously, the determination of hydrolysis products identifies the nerve agent except for its leaving group. However, the rather rapid elimination rate of the hydrolysis product from the organism (within several days) limits its use for retrospective detection of exposure.

Analysis of phosphylated butyrylcholinesterase (BuChE)

In principle, organophosphate-inhibited BuChE in human plasma is a persistent and abundant source for biomonitoring of exposure to organophosphate anticholinesterases. Polhuijs et al. (25) developed a procedure for analysis of phosphylated BuChE in plasma or serum samples, which is based on reactivation of the phosphylated enzyme with fluoride ions: this converts the organophosphate moiety completely into the corresponding phosphofluoridate, which is subsequently isolated and quantitated. As for analysis of hydrolysis products this approach identifies the organophosphate except for its leaving group. Moreover, the extent of the organophosphate poisoning can be determined in this way. Furthermore, based on the minimal concentrations of phosphofluoridate that can be analyzed in blood, it can be calculated that inhibition levels $\geq 0.01\%$ of inactivated BuChE (*i.e.*, trace level exposure) should be quantifiable. Evidently, by analyzing the inhibited enzyme instead of the uninhibited enzyme, inhibitor levels that are several orders of magnitude lower can be quantified. The method is limited by spontaneous reactivation and aging (i.e., loss of the alkyl moiety from the alkoxy moiety of the phosphyl group) of the phosphylated enzyme and by the natural life span of the enzyme. Application of this method to serum samples of the victims from the Tokyo subway attack and of the Matsumoto incident yielded sarin concentrations in the range of 0.2-4.1 ng/mL serum. Evidently, these victims had been exposed to an organophosphate with the structure $iPrO(CH_3)P(O)X$, presumably with X = F (sarin). A more laborious and qualitative method, reported by Nagao et al. (26) and by Matsuda et al. (27), is based on isolation and trypsinization of inhibited cholinesterases, subsequent treatment with alkaline phosphatase, followed by isolation, derivatization and GC-MS analysis of the released phosphyl moiety.

LEWISITE

Substantial stockpiles of the organoarsenical vesicant lewisite are present in the U.S.A. and in Russia. This may constitute a potential hazard for public health. The most generally applied method for determination of an arsenical is by atomic absorption spectrometry (AAS) after reduction of the compound to AsH_3. However, this will only provide an indication for the presence of the element As.

Lewisite will rapidly hydrolyze to 2-chlorovinylarsonous acid (CVAA) in aqueous environment such as blood plasma. Consequently, analytical methods mainly focus on the determination of CVAA. For instance, it was shown (28-30) that CVAA could be isolated from serum and urine after addition of 1,2-ethanedithiol, followed by extraction of the resulting complex, which could be analyzed by GC-MS. These methods, however, do not allow retrospective verification of exposure, since CVAA is rapidly excreted into the urine.

In view of the high affinity of arsenic for thiol functions, it can be expected that lewisite, as well as CVAA, binds to cysteine residues of proteins. It was found that 25-50% of the dose was associated with globin, after treatment of human blood with 20 nM to 0.2 mM of [^{14}C]lewisite (31). The CVAA residues could be isolated from globin after addition of 2,3-dimercaptopropanol (BAL), followed by extraction of the resulting lewisite-BAL complex, which could be sensitively analyzed by GC-MS after additional derivatization. The lowest detectable concentration of lewisite for in vitro exposure of human blood was determined to be 1 nM. A preliminary in vivo exposure was performed with guinea pigs. The amount of L1-BAL isolated from blood samples clearly decreased with increasing time after exposure, as should be expected. In the blood sample taken 10 days after exposure the amount of isolated BAL adduct had decreased to 10% of the amount at one day after exposure. The compound could only be detected in urine during the first 12 h after exposure, indicating the rapid excretion of unbound CVAA.

PHOSGENE

The pulmonary agent phosgene was used as a chemical weapon for the first time in WW I. Nowadays, it is an important intermediate for industrial production of insecticides, isocyanates, plastics, aniline dyes and resins, with an estimated yearly production of almost 1 billion pounds. Reliable diagnosis of exposure to phosgene other than observation of the developing edema by means of chest roentgenology is not available. It was recently found that phosgene binds effectively to albumin and hemoglobin upon in vitro exposure of human blood to [^{14}C]phosgene (32). *Inter alia*, phosgene appears to crosslink the lysine residues 195 and 199 in human serum albumin. A mass spectrometric method was developed for analysis of the tryptic digest containing this intramolecular lysine-lysine adduct, which enabled the detection of exposure of human blood to ≥ 1 μM phosgene in vitro. Whether this method can be used for assessment of in vivo exposure will be determined in due course.

CONCLUSIONS

- Adducts with macromolecules such as proteins offer long lived biological markers of exposure, possibly up to several months.
- Gas or liquid chromatography combined with tandem mass spectrometry is the method of choice for unequivocal identification at trace levels.
- The discussed analytical methods, with the exception of ChE inhibition measurements and immunoassays, cannot easily be performed, require expensive equipment and strongly deviate from methods for analysis of environmental samples.

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KEY WORDS
adducts, detection, lewisite, mass spectrometry, nerve agents, phosgene, sulfur mustard

44. NON-CONVENTIONAL TERRORISM: THREAT AND COUNTERMEASURES

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INTRODUCTION

Despite the demise of the Cold War, the world stability and security remains seriously threatened. The world is passing through a transitional phase. Power vacuum created by collapse of communism is being filled by the nationalist, ethnic and religious forces, which were long thought to be dormant.

Splintering of large and traditionally stable countries into smaller religious/ethnic areas, local and regional conflicts, economic disparity, mass movement of refugees, brutal and corrupt regimes and increasing porosity of national borders are fuelling frustration and desperation among masses which find expression in act of terrorism.

PURPOSE OF TERRORISM

The purpose, direction and focus of terrorism is fear. Broadly speaking, terrorism aims at accomplishing certain political, economic or psychological ends rather than to demonstrate sheer military power. However, contrary to popular belief, terrorism is not senseless destruction of life and property. Terrorist actions are neither spontaneous nor random.

These actions can serve a variety of purposes which may include; seizing political power, affecting public opinion and influencing the media, discrediting/disrupting the routine working of the government and provoking over-reaction or projecting an image of strength that far exceeds their numbers.

CHANGING FACE OF TERRORISM

Proliferation of Weapons of Mass Destruction (WMD), availability of qualified experts and free flow of information has made groups, even individuals, capable of decimating a large segment of population; a capability which was the sole preserve of major powers. With the globalization of transport network and erosion of technical barriers, use of nuclear, chemical, biological or radiological weapons by the terrorist groups is no more a far-fetched speculation but a reality.

The idea that terrorists could build and use WMD has been here for decades but few took the notion seriously until 1995, when Aum Shinrikyo sent a wake-up call to the whole world. This incident highlighted the concern that the use of WMD by terrorists was no more a fantasy but a real threat.

A growing number of incidents involving large-scale violence have taken place over the last decade. It is a common worry that the terrorists are becoming more inclined toward mass violence, rather than selective, limited attacks. Terrorists appear to be technically more proficient and less sensitive to political or moral values. This insensitivity to mass causalities, which was generally considered a traditional barrier, presents a dangerous scenario. This may make the WMD an attractive tool for the terrorists.

The typhoid poisoning (salmonella) of 750 people by the Rajneesh sect in Oregon, USA in 1984 and Aum Shinirikyo in Tokyo, where 12 died and over 5500 were injured, are the index of the future trend. With the Tokyo incident, a threshold was crossed. The taboo broken by the Aum Shinirikyo will have far-reaching effect on the future face of the terrorism.

NUCLEAR WEAPONS

Nuclear weapons can, principally, give terrorist organizations considerable advantage since they can cause mass causalities and can draw attention of worldwide media. Availability of fissionable material on the black market, economic crisis and deterioration of government control over radioactive material in some of the countries is another menace. The seizure of nuclear material in recent years has lent new credibility to the threat of nuclear terrorism as well. However, in view of the risk involved and the resources required to fabricate or steal a device, the choice of nuclear weapons by terrorists is less probable though not impossible. Nevertheless, a more likely threat of nuclear terrorism may be the radiological one i.e. the dispersal of radioactive substances to contaminate air or water or to render unusable a particular area or facility. Keeping in view the main focus of this symposium, ensuing discussion will be confined to chemical and biological weapons only.

MOTIVATION FOR CB WEAPONS

CB agents are here but the key question is: What motivates a terrorist to opt for these weapons?

- *Ease of Manufacture*

Small quantities of some of the CB weapons can be manufactured with little knowledge of chemistry and microbiology. Fabrication from readily available components by individuals with knowledge gained at the college level is possible. During most processing stages, only handling precautions are required.

"The technical requirements for culturing micro-organisms or producing toxins for use in bio-weapons are not particularly high - second or third year medical or micro-biology student would have enough laboratory experience - " US Congressional Office of Technology Assessment - 1992.

- *Lethality*

Even small quantities can have tremendous effect. Quantitative comparison of their lethal effects places biological agents foremost on the list followed by chemical agents. There is a wide range of causalities estimates associated with various CB agents but it will be highly misleading to extrapolate directly from individual lethal doses of a substance and estimate causalities resulting from mass attacks.

- *Production Cost*

They are cheap - a "poor man's atomic bomb". A program to produce a fissionable device would probably cost hundreds of millions of dollars. One report quotes "for a large scale operation against a civilian population, causalities might cost about $600 per square kilometer with nerve gas and $1 with biological weapons.

- *Detection Problem*

Non-detection by traditional anti-terrorist sensor systems provides another advantage. They cannot be revealed by metal detectors, x-rays machines or sniffing dogs as can guns, grenades and plastic explosives. Smuggling them through airports is as easy as the drugs, thus making the counter-measures more difficult. The ordinary look of a glass/plastic container can easily deceive security personnel.

- *Covert Nature of Operations*

CB weapons can be used covertly with little signature over periods of hours, even days. It is possible to devise methods to activate dissemination of CB agents with little signature. The first indication of a chemical attack may be when people start to collapse as happened in the Tokyo incident or, in the case of a biological attack, when people begin to develop symptoms of disease hours/days after an effective dose is ingested.

- *Ease of Spread*

Vapors and aerosols can be spread through large areas by natural convection. Ventilation systems in building/transportation facilities may actually augment the dissemination system by transporting CB agents far from the point of origin.

- *Monitoring/Neutralization – Resource Heavy*

CB agents require decontamination, tie up resources and increase media attention: they remain in the air as vapors or aerosols or settle on surfaces, thus becoming hazardous for a long time - months and years - if untreated. This needs continuous monitoring and decontamination, which is source intensive, time-consuming and tedious work.

- *Inadequate Response*

Government agencies are generally unprepared to face large-scale terrorist incidents. Level of knowledge, size of manpower and the type of equipment held by various civil agencies will mostly be insufficient to meet the enormous threat posed by CB agents.

- *Level of Escalation*

Action by the counter-terrorist organizations and subsequent reaction by terrorists can widen the scope of operations, thus drawing added media attention.

- *Flexibility in Target Selection*

Damage can be restricted to human beings leaving other materials and structure in tact. Biotechnologies are comparatively quite adaptable for use against small isolated targets, while retaining a capacity for attack on a larger target.

- *Reproduction of Organisms*

Biological agents are capable of reproducing; thus a small seed culture can produce a large quantity of agent so a much smaller quantity can infect a large population - also a disadvantage since control becomes difficult

- *Identification of Attack Difficult*

It is, sometimes, difficult for the victims to differentiate whether they are under attack or merely struck by a natural epidemic, as was the case of Salmonella poisoning in the US.

- *Time Lag*

The time lag between the release of a biological agent and its perceived effects on humans may cause difficulty in locating the terrorists or site of the actual attack.

- *Psychological Impact*

Impact of being exposed to CB agents is enormous and far lasting.

LIKELY TYPES OF AGENTS

There are numerous candidates for use by terrorists. However, keeping in view various practical considerations like availability, lethality, desired effects, flexibility in use etc., suggest that tularemia, shigella flexneri, salmonella, cyanides, ricin, Q fever, pneumonic plague are some of the serious contenders.

Smallpox can also be included in the list but possibility is remote since this is allowed to be stored only at two places (Center for Disease Control in Atlanta & Research Institute for Viral Preparation in Moscow)- both are secured sites. Probability of use of genetically engineered organisms, which is highly technical phenomenon, cannot be ruled out.

DELIVERY MEANS

Each means of dissemination has its own merits and demerits. Exact methods adopted by terrorists will depend on factors like terrorists' capability, purpose, effect desired at the target, technical proficiency, etc. Some of the means that may be adopted by terrorists are:

Contamination of food or liquids
Dispersal through vapors or aerosols within an enclosed/open area
Transmission directly through infected animals or inanimate materials like parcels/letters
Through direct human contact---ricin on umbrella tip
Direct conventional means/spread through explosion

COUNTER/REMEDIAL MEASURES

The objective of combating terrorism is to minimize effects, neutralize terrorist groups or render them ineffective and not necessarily killing them. Measures, which can be adopted to reduce probability of use of CB agents and to mitigate consequences when agents have been used, include:

- *Intelligence/Monitoring Operations*

Monitoring export/import of dual-purpose CB equipment, CB materials, etc.
Identification/monitoring of terrorist groups particularly those capable of acquiring or employing CB agents.
Intelligence collection on CB related activities of states sponsoring terrorism
Intelligence exchange of related information with friendly countries

- *Passive Protective Measures*

Detection being the first line of defense - full spectrum detection technologies
Detailed population evacuation plans particularly in major cities
Enhanced efforts towards R&D and stockpiling vaccines and other antidotes
Mitigation of Consequences
Developing quick and efficient decontamination capabilities
Capabilities in areas of domestic emergency response, hostage rescue, medical rescue
Creation of awareness among population
Coordination of international, national, regional and local assets to deal with the consequences
Isolation of contaminated population areas/material—though a difficult preposition
Rehearsing evacuation/emergency assistance plans
Information management/control

- *Operational Strategy*

Absolute determination to defeat terrorism - no compromise even in the face of the most severe intimidation/blackmail
Prosecution in the court of justice - top priority - no wavering
Tough measures against groups/states sponsoring terrorism
Unity of efforts to integrate actions of various national and international agencies
Restraint - premature action can be counterproductive if the action would interfere with intelligence development. which could compromise the security of plans
Avoid overreaction
Making the Verification regime of the Biological Convention more stringent and intrusive

CONCLUSION

Combating terrorism is a protracted conflict, which is void of physical frontiers or front lines. Adequate preparation at all possible levels not only can reduce vulnerabilities of intended victims but also degrade severity of post-attack effects.

The threat of CB terrorism can be curbed only if there is a complete consensus in the world community that it is an offence against humanity. Without joint and coordinated efforts by all countries, there are bleak chances that we can get rid of this menace.

There is an urgent need to upgrade our potential to effectively prevent and respond to terrorist incidents at the national as well as the international level. Let us realize the gravity of the situation before we all become hostage to this ever-growing menace.

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KEYWORDS

CB Terrorism, Unconventional Terrorism, Non-Conventional Terrorism, Counter Terrorism, Anti-Terrorism

46. IMIDAZENIL, A PROPOSED DRUG FOR THE TREATMENT OF CONVULSIONS IN ACUTE POISONINGS WITH ORGANOPHOSPHATES

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SUMMARY
Anticonvulsant and antilethal effects of imidazenil, a new imidazobenzodiazepine derivative, in soman acute poisonings were studied and compared to the effects of diazepam on mice and rats. It was stated that imidazenil in comparable manner to diazepam decreased intensity of convulsions, inhibited seizure bioelectrical activity and increased antilethal effectiveness of atropine and HI-6 in animals intoxicated with soman. These effects of imdazenil were noted in doses 5-10 times lower than those which influence the motor co-ordination when therapeutic doses of diazepam produce serious disturbances in motor co-ordination.

INTRODUCTION
Symptoms of acute poisonings with organophosphates (OP) include limbic seizures followed by general convulsions. This convulsive activity creates a problem for medical management and, if uncontrolled, can lead to brain damage. A combined regimen of prophylaxis and therapy, consisting of pretreatment with pyridostigmine and treatment with atropine and oxime, is now generally accepted as the most effective.

However, this combined treatment regimen does not appear to block OP-mediated seizure activity and concomitant motor convulsions. In experimental animals these seizures rapidly progress to status epilepticus and contribute to the profound brain damage (1).

Classic anticonvulsant drugs are not capable to increase significantly the antilethal effectiveness of atropine in OP intoxications. Therefore the first successful attempt to break convulsions in OP intoxications was introduction of benzodiazepines (BDZ) into the therapy (2,3,4).
BDZ, especially diazepam, could block or terminate OP-induced convulsions, reduce neuropathology, and enhance survival, especially when given in conjunction with carbamate pretreatment and atropine and oxime therapy.

Although neuropathology was significantly reduced compared with animals that did not receive diazepam the incidence and degree of protection afforded were never complete (5). Administration of diazepam before the start of convulsions prevented expression of the pathology, whereas, if diazepam was administered either at the start of or at various times after the initiation of convulsions the therapeutic benefit was quickly lost.

From the practical point of view it means that administration of diazepam must be initiated shortly after OP exposure, before the onset of convulsions, in order for any therapeutic benefit to be realized.

Diazepam alongside anticonvulsant activity has anxiolytic, sedative and myorelaxant properties which make it has potential to produce performance decrement when administered in anticonvulsant doses (6). This is of special importance when diazepam was administered to a person who was not intoxicated (e.g. as a result of a false chemical alarm). Therefore research should be continuing to find a better antidote against OP-induced convulsions and associated debilitation.

Partial BDZ receptor agonists are regarded as producing these side effects only in very high doses. Recently reported partial allosteric modulator of BDZ receptors, imidazenil (7) seems to be of special interest regarding the management of OP-induced convulsions.

Present study was performed in order to determine anticonvulsant and antilethal effects of imidazenil in acute poisonings with soman, a very toxic OP compound, and compare them with the effects of diazepam.

MATERIAL AND METHODS
Experiments were performed on Swiss strain mice (weighing 20-25 g) and Wistar strain rats (weighing 200-250 g) obtained from the Institute's own Animal Farm, with free access to standard food and tap water and in 12 h light/dark cycle. Experiments were carried out in accordance with the requirements of the Polish State Animal Protection Act (Scientific Procedures) and experimental design was approved by the local Ethical Committee of the Institute.

Soman (pinacolyl methyl phosphonofluoridate) was used as a model OP compound. It was dissolved in propylene glycol to 100 mg/ml and kept at 4°C. This stock solution was diluted to the desired concentration with redistilled water just before the experiment. Imidazenil and diazepam in crystal form were dissolved in a solvent proposed by Crankshow and Raper (8). Atropine sulphate and HI-6 in crystal form were dissolved in redistilled water.

Anticonvulsant efficacy was determined as:

(i) effects on intensity of convulsions induced in the mouse by s.c. administration of soman (200 μg/kg s.c.) and measured on Convulsometer (Columbus Instruments, USA). All animals in order to increase the survival rate received HI-6 (75 mg/kg i.p.). Experimental groups received imidazenil (2 mg/kg i.p.) or diazepam (5 mg/kg i.p.) immediately after the intoxication. Intensity of subsequent convulsions was measured at 10, 30, 60 and 120 min after the treatment and expressed in g/sec., and

(ii) effects on seizure bioelectrical activity of the brain due to administration of soman (180 μg/kg s.c.). Experiments were performed on rats with chronically implanted cortical stainless steel electrodes. Bioelectrical activity was registered every 5 min for 30 min using Grass Model 78 Polygraph. Four stages of intensity of seizure activity was determined according to Lowenstein et al (9): stage 1 - absence of spikes or sharp waves; stage 2 - discrete spikes and sharp waves on a normal background; stage 3 - high voltage spikes and sharp waves on a suppressed background; stage 4 - continuous or bursting high voltage spiking. Experimental groups received imidazenil (5 mg/kg i.p.) or diazepam (5 mg/kg i.p.). All animals received HI-6 (80 mg/kg i.p.) and methylatropine bromide (10 mg/kg i.p.) in order to increase the survival rate.

Antilethal efficacy was examined on mice as the influence of imidazenil (2 mg/kg i.p.) or diazepam (5 mg/kg i.p.) on the value of LD_{50} of soman given s.c. for 24 h observation and compared to the effectiveness of the standard therapy consisted of atropine (10 mg/kg i.p.) and HI-6 (75 mg/kg i.p.), using Thompson's method (10).

Statistical analysis was performed using Student's t-test for individual comparisons to evaluate the significance of means with the exception of antilethal efficacy when Litchfield-Wilcoxon method with the use of program set up by Tallarida and Murray (11) was employed. A p-value of 0.05 or less was required for significance.

RESULTS AND DISCUSSION

Doses of imidazenil or diazepam used in experiments were established previously (12).
Effects on convulsions intensity were depicted on the Figure.1. Effects of imidazenil were similar to those of diazepam. Both drugs diminished the intensity of convulsions due to soman (200 μg/kg) in the mouse immediately after application and their effect could be observed within 2 hrs.

Administration of soman in a dose of 180 μg/kg to the rats pretreated with HI-6 resulted within 10 min in a spike activity in the cortex (mean value of intensity stage 4), which lasted to the end of experiment, i.e. till 30 min. Administration of imidazenil or diazepam decreased the intensity of seizure activity (mean value for imidazenil 2.1, for diazepam 1.8) (Fig.2). Complete normalization of the record was not seen during the observation period.

Antilethal efficacy in the mouse was shown in the Table 1. Imidazenil in a dose of 2 mg/kg given as adjunct to standard therapy consisted of atropine (10 mg/kg) and HI-6 (75 mg/kg) resulted in an increase of the effectiveness of the therapy 3.6 times for 2hrs observation and 2.5 times for 24 hrs observation when effectiveness of diazepam (5 mg/kg) was 2.6 times and 1.5 times respectively.

Comparison of the effects of imidazenil and diazepam on performance of mice in Rota-rod treadmill was reported recently from our laboratory (12). Imidazenil was decreasing the performance ability in doses higher than 10 mg/kg when diazepam elicited strong effects on motor co-ordination in a dose of 5 mg/kg. To achieve the same level of effects as diazepam at 5 mg/kg a dose of imidazenil at 25 mg/kg was needed.

Diazepam is now commonly used for the management of convulsions in OP intoxications. In many countries diazepam is provided to military forces in special autoinjectors to deliver the drug i.m. by ordinary soldier as quickly as possible after the contact with OP (13).

However, when autoinjectors are used by non-professional people without real intoxication with OP (e.g. as a result of a false chemical alarm) a performance decrement and a decrease of fighting ability of the soldier due to sedative and myorelaxant properties of diazepam could be a real consequence.

Our present results indicate that effectiveness of imidazenil in the management of soman-induced convulsions is very close to the effects of diazepam. Effects of imidazenil on soman-induced seizure bioelectrical activity of the brain are also comparable to those of diazepam. And antilethal effects are even higher than those of diazepam.

However, diazepam in therapeutic doses (from 2 mg/kg in the mouse) produces very strong disturbances in motor coordination. Such an effect was observed after the administration of imidazenil in a dose of 25 mg/kg, i.e. > 10 times higher than therapeutic dose (2 mg/kg in the mouse). This is a very great practical advantage of imidazenil.

However, imidazenil in any country is not approved and registered as a drug. Imidazenil, according to Costa and Guidotti (14) is considered as a potential antiepileptic drug of new generation. If further studies, especially those concerning the chronic toxicity, confirm the positive initial opinion and imidazenil would be registered it could become a drug of choice for the management of convulsions in OP intoxications.

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KEYWORDS
convulsions, diazepam, imidazenil, organophosphates, Soman

FIGURES AND TABLES

Figure 1. Effects of imidazenil or diazepam on convulsions due to soman (200 µg/ kg sc) intoxication in the rat.

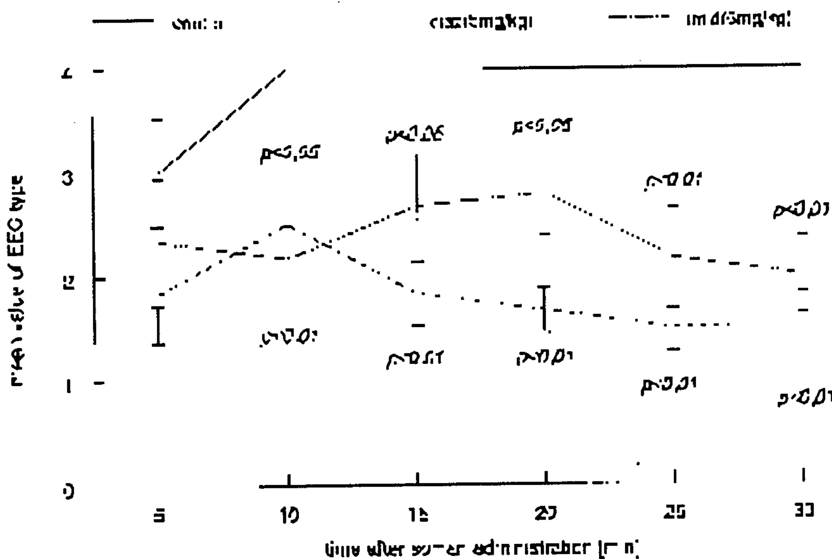

Figure 2. Effects of imidazenil or diazepam on the soman (180 μg/ kg sc) induced seizure bioelectrical activity of the rat brain.

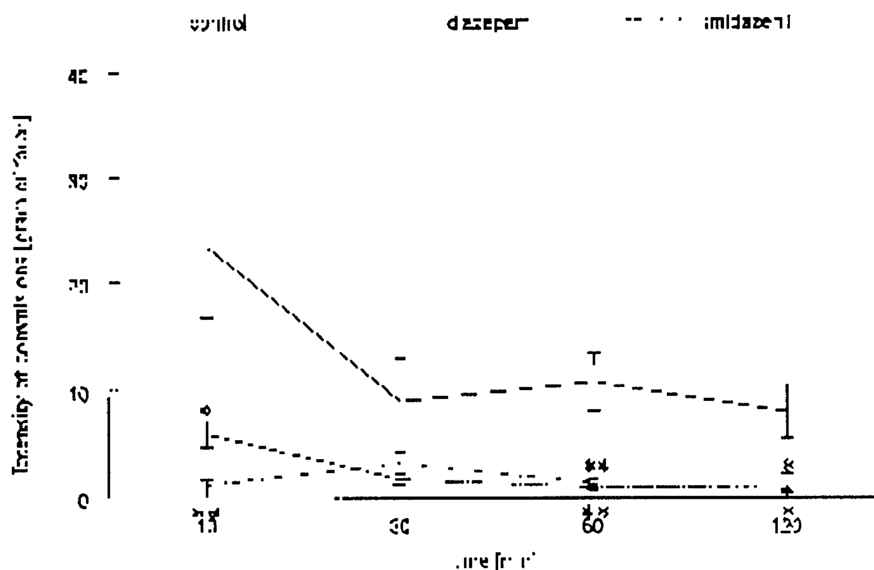

Table 1. Antilethal effects of diazepam (5 mg/kg ip) or imidazenil (2 mg/kg ip) given together with atropine (10 mg/kg ip) and HI-6 (75 mg/kg ip) in soman (μg/ kg sc) intoxication in the mouse.

	LD 50	TE *
soman	137(126-150)	1
soman + atropine + HI-6	856 (609-1204)	6.2
soman + atropine + HI-6 + diazepam	1287 (808-1832)	9.4
soman + atropine + HI-6 + imidazenil	2140 (1640-290)	15.6

* Therapeutic efficacy = LD 50 with treatment : LD 50 without treatment
In parentheses confidence limits

47. PHARMACOKINETIC COMPONENT OF THE MECHANISM OF ACTION OF DECORPORATORS

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ABSTRACT
The decorporation rate of heavy metal ions from living body can by increased following their complexation by polyanions, called chelators. The limitation in efficiency of all chelators arises from some pharmacokinetic particularities: poor absorption, fast elimination and lower penetrability through cellular membrane.

Since repeated doses can lead to modifications of the plasma levels profile, we evaluated the pharmacokinetics of calcium edentate after multiple i.m. administration and after single i.v., i.m. and oral administration, in healthy volunteers. The pharmacokinetic model appears to be bicompartmental, in spite of the fact that the ion cannot leave the central compartment. The repeated doses did not induce an accumulation, which was considered another argument to reject the idea of the access of edentate ion in a "deep compartment". In order to explain the apparent bicompartmental behavior we conceived a pharmacokinetic model of "total edentate ", including Ca, Zn and Cu complexes.

INTRODUCTION
The decorporation rate of heavy metal ions from living body can be increased following their complexation by hydrophilic polyanions, named chelators. Resulted compounds have different properties than the ions in blood. The most useful between these is the higher diffusion coefficient, which explain the increase of renal elimination. The applications of this pharmacokinetic modification concern both treatment of professional intoxications [1-3] and decorporation of some radionuclides [4,5].

The limitation in efficiency of chelators arises from some pharmacokinetic particularities: poor absorption, fast elimination and lower penetrability through cellular membrane. Consequently, repeated doses, at short intervals between administrations are necessary for obtaining significant therapeutic results. Since, on the other hand, repeated doses can lead to modifications of plasma levels profile of chelators as well of their complexes, it was considered that it deserves to evaluate the pharmacokinetics of calcium edentate after multiple i.m. administration in healthy volunteers in comparison with the pharmacokinetics after single i.v., i.m. and oral administrations.

EXPERIMENTAL METHODS
Four normal volunteers received (by i.m.) five doses of 10 mL sodium salt of ethylenediaminetetraacetic acid ($CaNa_2EDTA$ - calcium edentate) 10 % solution, every 12 hours. Blood samples were prelevated at 0, 30, 60, 90, 120, 180, 240 and 300 minutes after the first and the fifth administration. Two of the volunteers also received the same dose intravenously. After erythrocytes separation, plasma levels of EDTA were evaluated using a Mettler DK phototitrator, at $\lambda = 570$ nm, after transformation of its complexes with Ca, Fe, Cu and Zn in the more stable Bi complex.

RESULTS
Pharmacokinetic after repeated i.m. administrations
Looking at the graphical representation of the complete set of data, we observe a large enough inter-variability, as can be seen from Figure 1, but at the same time, a global coherence can be assumed. After a rapid rise, the concentration attained values of 25 - 40 μg/mL, the time of maximum being between half an hour and one hour. Since the analysis of variance (ANOVA) confirmed the hypothesis of the equality of intra and intervariability of the main pharmacokinetic parameters (absorption constant, area under curve, elimination constant), all further analysis started from the mean values plasma levels corresponding to same administration (first and fifth respectively).

The graphical representations of the mean plasma levels after first and after fifth dose are presented also in Figure 1. The elimination phase appears to be practically identical, a normal result in the case of dose-independent pharmacokinetic. A greater maximum concentration and a smaller t_{max}, after the fifth dose, though normal from the point of view of a phenomenological approach, were not statistically significant.

After a rapid absorption, the concentration attained values of 25-50 μg/mL. Half-time was found to be approximately one hour, in agreement with values of somewhat less than one hour, accepted by pharmacological literature [6].

Fitting the experimental data with the solution of a monocompartmental model led to a very good approximation. Further, fitting both mean curves for first and fifth administration with the same solution of a bicompartmental model also led to good results. As can be seen from Table 1, the computed coefficients of transfer between central and "deep" compartment were meaningless from the phenomenological point of view: $k_{12} >> k_{21}$ signifies a higher affinity for deep compartment than for blood compartment, which is difficult to accept in the case of hydrophilic substances.

PK after oral administration

Computed pharmacokinetics parameters using a monocompartmental model are presented in Table 1. It is notable that Cmax ≈ 1 - 2 μg/mL, a value more than 20 times lower than that after i.m. administration. Further, $T_{1/2} \approx$ 2h, seems to be greater than after i.m. administration. Computed bioavailability was less than 10 %.

A more in detail examination reveals that we have at half-time an "initial" value of the same order as that after i.m. administration, but in the "final" part of the curves the value of $T_{1/2}$ increases to almost double values.

Pharmacokinetics after i. v. administration

Surprisingly, the result after intravenous experiment rejected the idea of a single compartment: the concentrations cannot be described by a single exponential since, as can be seen in Figure 2, their logarithm cannot be fitted with a line.

DISCUSSION

Differences in computed pharmacokinetics parameters after i.v., i.m. and oral administrations are difficult to explain since distribution and elimination phases are independent of the means of administration. If we put three curves of one volunteer on the same graphic, as can be seen in Figure 3, noting the great differences in the mean plasma concentrations, we think also of a possible "dose-dependent " pharmacokinetics.

Another problem, which appeared after the examination of results, concerns the pharmacokinetic model, followed by edentate ion. The problem is directly connected with its efficiency as a heavy–metal antagonist. A two compartmental distribution would imply the possibility of removal of "circulating" metal, as well as a part of fixed metal. As is commonly seen with heavy metal poisoning, effectiveness of treatment diminishes very rapidly with an increasing delay between exposure and the initiation of therapy, which pleads for water compartment as the site of antagonist action.

Pharmacokinetic model: one compartment or two compartments?

Arguments for 1 compartment

- Phenomenology: EDTA doesn't enter in cells and in lipids
- Fitting experimental data through application of a bicompartmental model leads to an excellent fitting but
- the huge values of computed parameters for transfer between blood and "second compartment" are meaningless.

Arguments for 2-compartmental model

- i.v. data cannot be represented by an exponential but can be "well" fitted with a biexponential curve
- the differences between $T_{1/2}$ after oral and i.m. administration leads to the idea of a "distribution" phase.

All these contradictions and dilemmas can be explained if we make the hypothesis that in a first time interval, part of the Ca in the complex with edentate is replaced by other oligoelements. This results in the formation of more stable complexes and, in a second phase, new complexes are eliminated by renal way with different lower rate than the Ca complex.

Such a way, a "pseudo" bicompartmental model - with the complexes of EDTA with oligoelements (Cu, Zn, Fe, Mn) as the second compartment is to be considered. The justification of this splitting of the central compartment is supported by the differences in the stability and elimination rates of EDTA complexes.

The differences in elimination between the oral and i.m. case could be explained by the differences in the plasma levels. At high concentrations, the effect of the complexation of oligoelements by edentate is negligible. On the contrary, after oral administration the levels of edentate are of the same order with Zn, which "consumes" a significant part of chelator. To some extent, even the binding of a second calcium ion is possible. However, in these complexes both calcium and oligoelements can be replaced by heavy-metal ions, which give more stable complexes with edentate. Since all these reactions take place in water compartment, for longer periods the kinetics of decorporation is determined by the rate of transfer of heavy-metal ion from other compartments to the blood.

Last but not least, poor oral absorption can be at the same time a prolonged absorption, but it is clear that this phenomenon is absent in the case of i.v. administration.

Otherwise, the idea of a second compartment is not entirely new. An extensive study of the metabolism, pharmacokinetics and toxicity of chelators in rats [7] revealed a multiexponential elimination: ≥ 99 % of the chelators are cleared with a half-time while the remaining fraction has a much longer half-life (≥ 20 hours). The second compartment was considered a "pseudocompartment"; the slow term is due to binding of chelators by the plasma proteins.

CONCLUSIONS

Repeated daily doses of 1 g of $Na_2CaEDTA$ do not assure sufficient plasma levels for more than some hours for a "continuous" decorporation and do not lead to an accumulation.

Oral bioavailability of EDTA solution is lower than 10%, but an apparent doubling of the half-time could be significant in intoxications with heavy-metal ions having a greater affinity face to edentate than the oligoelements.

Pharmacokinetics present a "pseudobicompartmental" behavior and "dose dependence" which can be considered a consequence of a partial substitution of Ca with oligoelements.

KEYWORDS

decorporation, chelators pharmacokinetics

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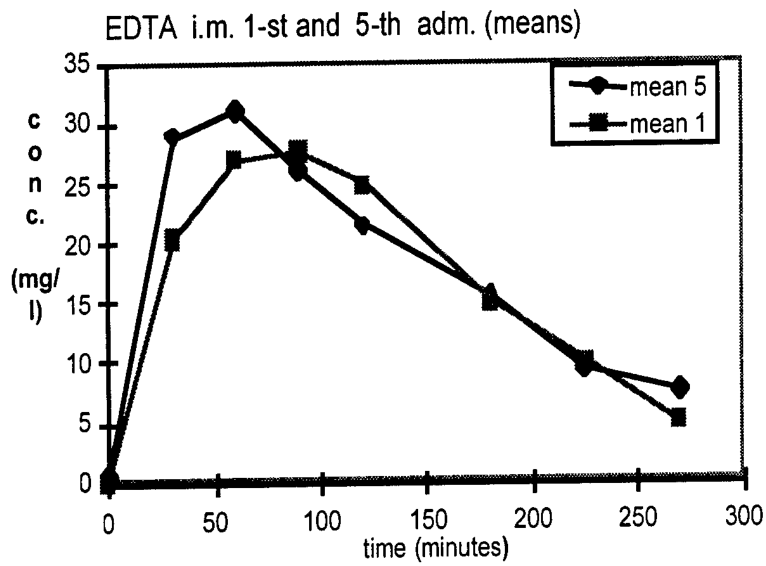
FIGURES AND TABLES



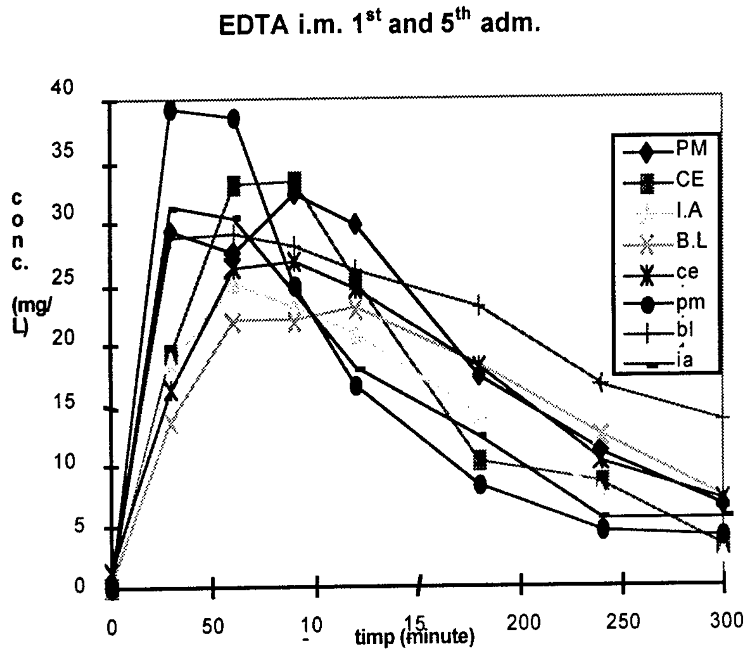


Figure 1. Plasma levels of edentate ion after i.m. administration

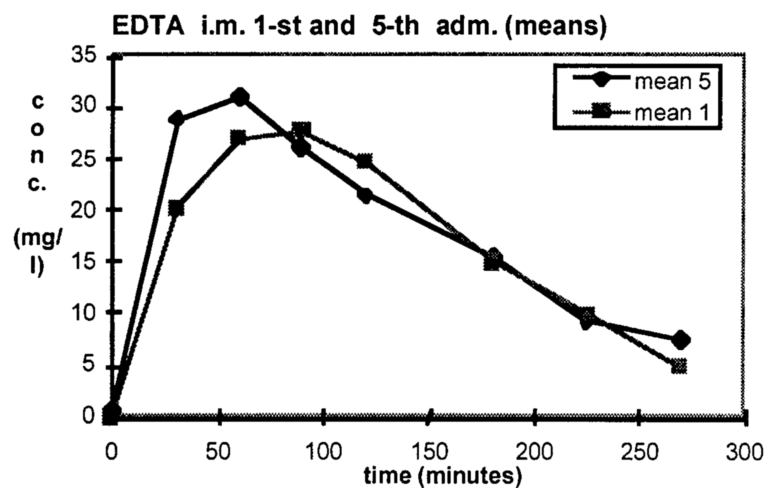


Figure 2. EDTA concentration after i.v. administration (logarithmic representation).

Table 1. Parameters and Coefficients

Parameter	Value	t50%	Coeff Zi	AUC	Coeff Ai
Name	[1/min]	[min]		[%]	
b1	13.0	0.0533	0.920	1.3	-0.00105
b2	0.148	46.9	0.0803	98.7	-165.
k31	1.06				
k1e	0.182				
k13	11.8				
k01	0.0148	47.0			165

Table 2.

VOLUNTEER	C_{MAX} (µg/mL)	T_{MAX} (minutes)	$T_{1/2}$ (minutes)	k_a (min^{-1})	k_e (min^{-1})
CM	1.53	180	123	0.0056	0.0056
TM	1.95	180	63.1	0.0111	0.0109
CE	0.77	180	476	0.0143	0.0014

48. EPIDEMIOLOGICAL APPROACH OF BIOLOGICAL CRISIS: BY CRIMINAL USE OF *BACILLUS ANTHRACIS*

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INTRODUCTION

Anthrax is primarily a disease of domesticated and wild animals, particularly herbivorous animals. Humans become infected incidentally when brought into contact with diseased animals, their hides, hair or their excrement. Many species of animals and birds can acquire the disease naturally (1). From earliest historical records until the development of an effective veterinary vaccine, together with the subsequent therapy with antibiotics, the disease was one of the main causes of uncontrolled mortality in cattle, sheep, goats, horses and pigs worldwide.

Humans almost invariably contact anthrax directly or indirectly from animals. The disease is still enzootic in most countries of Africa and Asia, South and South-East Europe, in some areas of the American continent and Australia. The disease still occurs sporadically in many other countries (2).

Bacillus anthracis is associated with biological warfare because of the gravity of the disease in human, especially pulmonary form. A huge quantity of spores released in the air, with criminal intent, is expected to be followed by a substantial devastation in human and animal communities within selected targeted areas. The facile way in obtaining spores, with minimal laboratory equipment, made *Bacillus anthracis* a very dangerous weapon that can also be used in terrorist attack.

Research on anthrax as a biological weapon began more than 80 years ago. Today at least 17 nations are believed to have offensive biological weapons programs. It is uncertain how many are working with anthrax. Iraq has acknowledged producing and weaponizing anthrax (3). The Aum terrorist group from Japan, responsible for the release of sarin in a Tokyo subway in 1995, dispersed aerosols of anthrax and botulism throughout Tokyo on at least 8 occasions (4)

The accidental aerosolized release of anthrax spores from a military microbiology facility in Sverdlovsk in the former Soviet Union in 1979 caused an epidemic of pulmonary anthrax, with 68 lethal cases from 79 recorded victims. That accident demonstrated without any doubts the devastating effects of using aerosol with anthrax spores as a weapon (5).

Anthrax spores were weaponized by the United States before the US offensive program was terminated in 1971-1972. The most recent evidence of this threat is that in 1995 Iraq admitted to weaponizing Bacillus *anthracis* (6).

THE ORGANISM

Bacillus anthracis is a worldwide bacillus, with reservoir in the soil, and sporulating in specific conditions. *B anthracis* exists in the infected host as a vegetative form and in the environment as a spore. *Bacillus anthracis* is a non-motile, Gram-positive, spore-forming capsulated bacillus, which is both an aerobe and facultative anaerobe.

The vegetative form produces a toxin that causes gelatinous edema and hemorrhage in animal tissue. There are three distinct antigenic components: a somatic protein, a capsular polypeptide and a somatic polysaccharide. The protein somatic antigen stimulates immunity in most animals, is present in the edema of anthrax lesions, and is neutralized by the anthrax antiserum. Spores form only if the bacteria are exposed to air. The spores are very resistant and will resist dry heat at 140°C for one to three hours and 100°C moist heat for five to ten minutes. *B. anthracis* grows on sheep blood agar aerobically.

Bacterial identification is confirmed by demonstration of the protective antigen toxin component, lysis by specific bacteriophage, detection of the capsule by fluorescent antibody and virulence for mice and guinea pig. Additional confirmatory tests to identify toxin and capsules genes by polymerase-chain-reaction (PCR) h[illegible] also been developed as research tools (7).

The most common way in the nature of forming spores is when the carcass of a dead animal by anth[illegible] opened by other animal or by man. Anthrax spores survive and remain viable in the environment, e[illegible] adverse conditions, for decades. When spores meet optimum conditions, they change into the vegetati[illegible] and infect the host, which will develop the disease.

EPIDEMIOLOGY

Anthrax occurs worldwide. The organism exists in the soil as a spore. Animals, domestic or wild, become infected by ingestion of spores with grass, contaminated land or eating contaminated feed. The grazing animals are the most susceptible to infection. Cattle, sheep, goats, and horses are the most frequently infected animals. However any animal, could theoretically be infected. In animals the portal of entry is the mouth and intestinal tract by the ingestion of spores on vegetation (Figure 1) (2).

In humans, infection is acquired from animal sources through the skin - cutaneous anthrax or malignant pustule, or by the intestinal route from using infected animals as food - oropharyngeal or gastrointestinal anthrax, or by inhalation of spores into the lungs - pulmonary anthrax or woolsorter's disease. Pulmonary anthrax occurs in the textile and tanning industries among workers handling contaminated wool, hair and hides (8). The largest reported epidemic of human anthrax occurred in Zimbabwe between 1978 – 1980, with about 10,000 cases. Most of these were cutaneous, with a few gastrointestinal and pulmonary cases (7). In Romania morbidity by anthrax for the last ten years is around 0.035 cases at 100,000 people, with only cutaneous cases. A national vaccination program in domestic animals is performed under control of national veterinary authorities. Anthrax has little potential for person-to-person transmission; standard precautions are thus adequate for health-care workers treating anthrax patients (9).

THE DISEASE

There are three forms of disease, following the way of entrance of the spores in the human body. The pulmonary form is the most severe, with high mortality, over then 95% after onset of clinical symptoms.

In the pulmonary tissue spores are phagocytized by macrophages and transported to hilar and mediastinal lymph nodes. The spores germinate into vegetative bacilli, producing a necrotizing hemorrhagic mediastinitis.

The disease starts with a prodrome featuring fever, malaise and fatigue. After two or three days onsets a severe respiratory distress with dyspnea, stridor, diaphoresis, and cyanosis. Toxico-septic shock, meningitis are common complication and, unfortunately death follow within 24-36 hours.

Once symptoms of pulmonary anthrax appear, treatment is almost ineffective (10).

Treatment and Prophylaxis

Treatment and prophylaxis are based on antibiotics and active immunization.

Classic penicillin is the most effective and is the drug of choice, but some resistant strains have been described. Other effective antibiotic regimens are with tetracycline, doxycyline and erythromycin, for the penicillin allergic patients. Chloramphenicol, gentamicin and ciprofloxacin are active on natural strains. Inhalational, oropharingeal, and gastrointestinal anthrax should be treated with large doses of intravenous penicillin, two million units given intravenously every two hours, with appropriate vasopresors, oxygen, and other supportive therapy.

Active immunization is the main prophylaxis measure, both in animals and humans. Vaccines for veterinary use are prepared from live strains and are used worldwide (2, 7, 8, 10).

There are only a few human vaccines. Russia and China use live attenuated strains. These vaccines can be administered by aerosol, scarification or subcutaneous injection. The United States and British vaccines are filtrates from two different anthrax strains. These vaccines are made by a combination of the three fractions of anthrax toxin; the main component being a protective antigen (PA).

The British vaccine consists of alum-precipitated toxin proteins and has larger amounts of edema factor (EF) and lethal factor (LF) than the US vaccine. The United States vaccine consists of a culture filtrate from the toxigenic, non-encapsulated strain of B. anthracis that has, in addition to PA, small amounts of EF and LF. The US vaccine uses aluminum hydroxide to absorb PA and to serve as an adjuvant to increase the potency; the adjuvant is believed to stimulate humoral but not cell-mediated immunity (11). Vaccination of US troops with this vaccine is in progress and the results are encouraging (3, 12).

There are two different situations: **pre-exposure prophylaxis**, made by vaccination, as it was mentioned above and, **post-exposure prophylaxis**, that has two goals: a) to prevent infection after exposure to spores and. b) to prevent the onset of clinical symptoms caused by anthrax toxin. In the a) case antibiotics are indicated as prophylaxis regimen. To prevent the onset of clinical symptoms we consider administration of anti-anthrax immune serum very useful. In our opinion, this policy, despite all inconveniences, such as anaphylaxis reactions, is a life saving policy, especially if it is applied as soon possible after exposure.

PICTURE OF EPIDEMIC OF PULMONARY ANTHRAX BY CRIMINAL RELEASE OF SPORES OF B. ANTHRACIS

In 1970, a World Health Organization (WHO) committee estimated that casualties following the theoretical aircraft release of 50 kg of anthrax over a developed urban population of 5 million would be 250.000, 100.000

of whom would be expected to die without treatment (13). (Aspects of Chemical and Biological Weapons. WHO, Geneva, 1970: 98).

A 1993 report by the US Congressional Office of Technology Assessment asserts that between 130,000 and 3,000,000 deaths could occur after an aerial release of 100 kg of anthrax spores upwind of the Washington. DC area - lethality matching or exceeding that of a hydrogen bomb (3).

The theoretical evaluation of the cost of a biological attack with anthrax suggested a cost of $26.2 billion per 100,000 persons exposed (14).

Evolution of the outbreak can be split in 4 periods:

Attack or release of the anthrax spores and incubation period is silent and probably unexpected. Incubation takes about 1-6 days, but can be prolonged. It depends on quantity of spores inhaled (the infectious dose is estimated at 8,000-10,000 spores). The biological attack may have no external trace and the devices used for delivery are varied, from classical devices (bombs, missiles) to unconventional devices such as spraying. aerial delivery, using the crowded places for spraying etc. No clinical symptoms are observed in the incubation period.

Onset of the disease, the first cases and the diagnosis. The initial symptoms are very common for many diseases: fever, malaise, nonproductive cough, fatigue, etc. Except for possible intelligence information regarding the threat of a biological attack, the first cases will be misdiagnosed because of several factors such as: mimicry of other diseases including the flu, lack of clinical and epidemiological data and so on.

Very probably, the cases' onsets at the beginning of the epidemic will be lost due to medical reason – no correct diagnosis, no adequate treatment.

Two or three days after its clinical debut, a huge number of patients will need very sophisticated medical assistance, due to their condition. That number is estimated to be around 70-80% of all victims. This situation will result in an overwhelming use of medical resources. Due to that, many patients will be lost. However. even with the greater medical assistance, mortality in pulmonary anthrax is estimated to be more than 80% (Figure 2). (14).

In a city with 100,000 contaminated habitants, if 50% are infected it is estimated that in 3-4 days. about 15-20,000 people will require intensive care assistance and of these, more then 60,000 will be lost (Figure 3).

Diagnosis and specific countermeasures. Once the correct diagnosis is made, everything becomes clear. despite the great difficulty in managing the situation. Specific measures can be taken in order to limit the disaster.

The end of the epidemic and recovery period depends on the extent of the epidemic in the area of responsibility and on the average of people and animals affected by disease. The economic power in the region and the external support are essential to implement the policy of recovery in the area.

Action To Be Taken:

In the first stage, in the absence of any information, no action can be taken. This is the worst situation because without preparedness and prophylactic actions, the onset of the epidemic will be devastating.

Active immunization and prophylactic medication should be commenced in the exposed population as soon as possible if any suspicion arises and a primarily medical investigation suggests an attack with biological weapons.

The second phase is crucial in the evolution of the event. Many situations could occur. and after the onset of clinical disease the evolution of the epidemic becomes more and more complex. The large number of patients with bed-confined conditions and the development to acute respiratory failure requires a huge medical support that could very quickly deplete the stock of medicine and consumables, if is not quickly resupplied.

Rapid overcrowding of medical facilities, lack of qualified personnel, panic in the population and rapid exhaustion of support personnel are in our opinion factors that greatly complicate the situation. After people start to die, safe burying of the deceased remains, to avoid the accidental forming of spores. becomes a very sensitive problem. Veterinary service has to manage the disease in animals.

Once the diagnosis is made, all measures become coherent and more efficient. Specific treatments with antibiotics and vaccinations are expected to reduce the number of fatalities. Unfortunately, the evidence that we have shows a high mortality after the disease is manifest. Hygiene and sanitation are very important. All animal cadavers must burned or buried very deeply.

The period of recovery depends on community's capability to develop and to support the reconstruction in the affected area.

CONCLUSIONS

Management of an epidemic with anthrax is very complex and without doubt requires the common effort of the entire community with national or international support. The critical time of action is very short and could be overtaken by rapid evolution of the epidemic. Even then it is mostly therapeutic and prophylactic measures. Historically, we consider that immune serum therapy is very useful in clinical anthrax because the symptoms are induced by toxin. A similar policy is taken in tetanus or diphtheria treatment. Before antibiotics, the mortality in anthrax was significantly reduced with immune serum therapy to about 5%, in the gastrointestinal form (8). Modern methods could avoid many inconveniences of serum therapy with the benefit to reduce mortality.

KEYWORDS

Bacillus anthracis, anthrax, epidemiology, prophylaxis, biological weapon

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FIGURES AND TABLES

Figure 1. Transmission of Anthrax

(World Health Orgnization- Guidelines for the Surveillance and Control of Anthrax in Humans and Animals. 1998.)

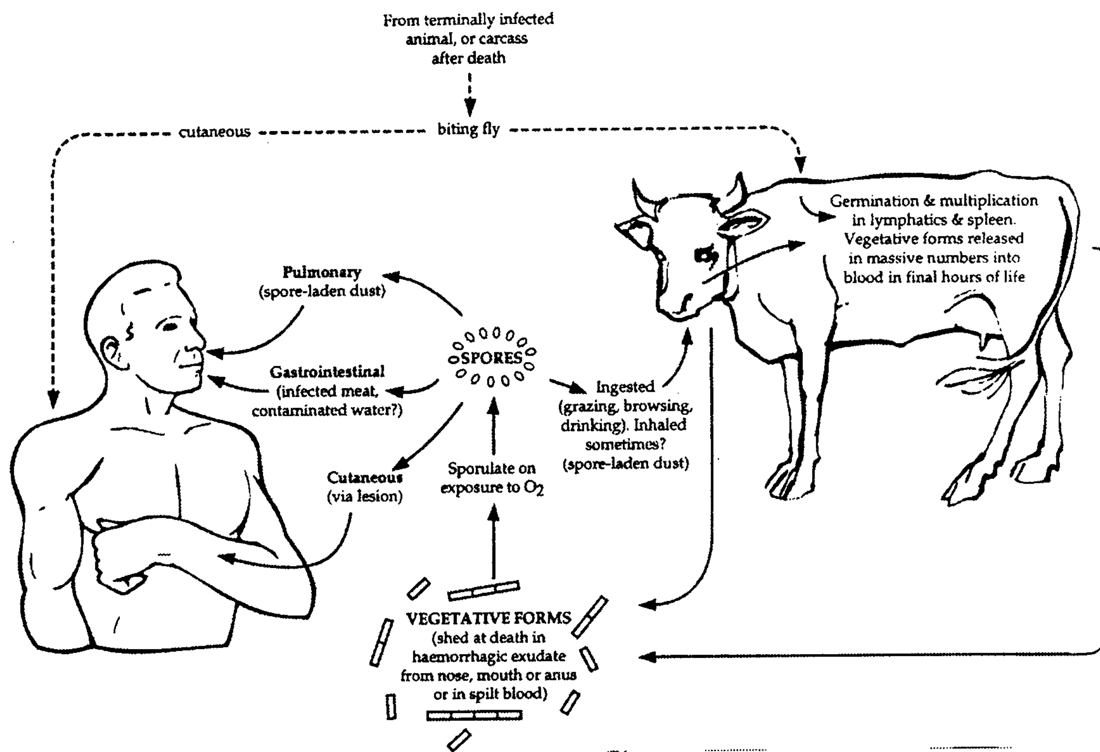

Figure 2. Epidemic Curve of Cases by Day of Exposure and Rate of Fatal Cases. Based on estimation of Kaufmann et al, Emerg.Inf.Dis,1997,3(2):84), modified

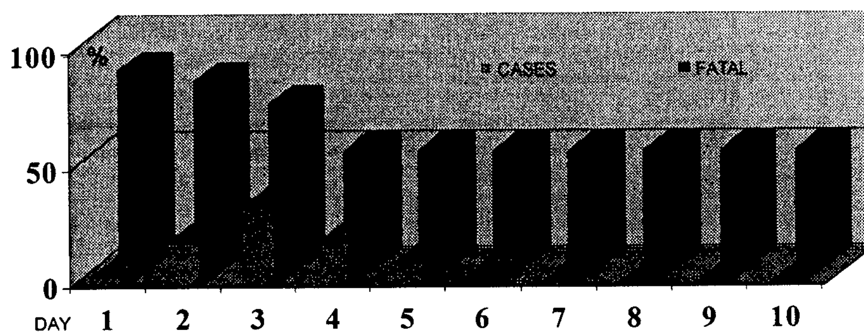

Figure 3. Evolution of Morbidity and Mortality (based on 1000 infected persons)

49. CREATION OF MODERN SYSTEMS OF WARNING OF POPULATION AND PERSONNEL DURING EMERGENCY AT THE SITES OF CHEMICAL WEAPONS STORAGE AND DESTRUCTION IN RUSSIA

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INTRODUCTION

During large-scale emergency situations, in which de-encapsulation of a large number of CW containers occurs, or with heavy damage of CW storage facilities, the main way to protect the population and personnel at the CW storage sites in the Udmurt Republic, (on which territory there are two poisonous substance (PS) storage sites: in the city of Kambarka (Lewisite) and in the settlement of Kizner (phosphor-organic substances - POS)), is to carry out certain measures directed toward evacuating the population and the personnel [1]. If these measures are executed in proper time, the number of possible victims will be decreased greatly.

The existing warning systems at CW storage sites are obsolete and cannot be used for the purpose of immediately warning the population inhabiting the CW storage zone. Nor can they be used for the maintenance of the facilities of CW destruction, the construction of which is supposed to take place next to the storage buildings [2]. Therefore, the main task connected with carrying out the works on CW destruction in Russia, is to create a modern system to warn the population and the personnel of the CW storage sites. For this we are involving the updated achievements in the sphere of PS analysis, computerized methods of event development prediction and updated means of electronic communication.

ANALYSIS AND DISCUSSION

An updated automated warning system for the population and personnel should become the main focus of the entire system of collective safety of the created facilities for CW destruction. In this case it should include the following:

1. CW destruction facility;
2. Sanitary zone of the CW destruction facility;
3. Part of the zone of the protective measures outside the sanitary zone of the CW destruction facility; As far as the CW destruction facilities are supposed to be located in the radius not more than 4-5 km from the storage facilities [3], this system should maintain:
4. CW storage facility;
5. Sanitary zone of the CW storage facility;
6. Part of the zone outside the sanitary zone of the CW storage facility.

According to the project [4] which is being prepared, the responsibility for carrying out the protective measures is distributed among 3 control organs: items 1, 2 - the sphere of responsibility of the head of the CW destruction facility, items 4, 5 - the head of the CW storage facility, items 3, 6 - the head of the local authority. Meanwhile large-scale emergency situations present certain danger to all above mentioned facilities and zones. Therefore the security systems of the CW storage and destruction facilities cannot be of local importance only; they should be a part of the entire security system.

The whole security system for large-scale emergency situations should consist of the following constituent parts:

1. Sensors and methods of analysis of the chemical contamination:

a) PS sensors and analyzers, working continuously in the mode of real time, located in the CW storage and destruction facilities. These devices should posses high sensitivity to detect large concentrations of PS; the ability to give the warning signal automatically and they should be designed as fireproof and explosive-proof devices. Their main function in the security system is to give the signal of chemical alarm in proper time.

b) sensors and gas-analyzers, working continuously in the mode approximated to real time and having the ability to automatically analyze relatively low concentrations of PS. The location of these devices should be mainly in the sanitary zone around the CW storage and destruction facilities. Their function will be the operative correction of the mathematical model for prediction of the situation.

c) gas-analyzers for detecting low and maximum allowable concentrations of PS. These devices should be located in the mobile labs and the analysis of PS is to be carried out on the borderline of the sanitary zone and the zone of

the protective events. The analysis of PS in this case should be done within 10 - 15 min. The results are also used for the correction of the mathematical model for situation prediction.

The majority of gas-ion-analyzers and military devices may be included in the group of devices a) and b). The devices of group c) are chromato mass-spectrometers or the devices operating on the principle of cholinesterase conversion [5].

2. The computerized system to prediction the developing situation and to confirm the decisions on carrying out the measures to protect the population and personnel. The system involves a mathematical model of PS cloud spread with the use of geo-informational systems for defining the district and performs prediction and tracking of the propagation of maximum-allowable and lethal PS concentrations front in real time mode. The system allows for adoption of decisions on warning and evacuation and to control the works on elimination of the emergency situation consequences performed by special groups of people. The companies BRUNNEWTECH (Denmark) and TEKLA (Finland) have developed similar computerized systems. At the present moment in Russia such systems are still being studied and developed [6, 7].

Such a computerized system is equipped for continuously receiving initial data from the automated meteorological service, and for receiving the more exact initial information it can be equipped by video monitor.

The computerized system is connected with devices for giving warning signals and with various services for protection of the population and personnel.

The information given by the sensors a) and b) and by the lab c) is used for correction of the applied mathematical model.

The use of the mathematical methods for assessment of the PS vapor spread at the levels of maximum allowable concentration in the mode approximated to real time is very important as far as Russian adopted maximum-allowable concentration values are even lower then those in foreign countries. In Table 1 the values of safe concentrations is adopted in Russia and the USA [8], the levels of sensitivity of some devices and the time necessary for PS detection are presented [5,9].

From Table 1 it can be seen that the existing devices are not enough to realize the detection of PS concentrations at the level of maximum-allowable concentration in real time mode or in the mode approximated to real time. The assessment of the movement of the front of maximum-allowable PS concentrations in the mentioned modes of time can be performed only on the basis of mathematical methods. At the same time the determination of the movement of this front necessarily influences the decision to evacuate the population.

3. The warning systems (sirens, radio devices, television, monitors) connected with the main computer are used for giving alarm signals and receiving on-line information from the main computer.

During a large-scale emergency situation the system works as follows: After the emergency has taken place at the CW storage or destruction facility the signal is given together with the initial data to the local protective system of the facility and to the whole security system. At the same time the system receives the information from the chemical contamination sensors, located in the sanitary zone of the facility. According to the initial data received from the chemical contamination sensors and from the meteorological service, the assessment of the situation is done and the prediction of its development is performed by the computerized system dealing with situation development prediction and confirming decisions. The mobile lab is given the signal to move by a certain route. The mobile lab, which has the preliminary information about the emergency situation, begins to perform the chemical contamination analysis even before receiving the information from the computerized system. The information received from the sensors for detection of chemical contamination and from the mobile lab together with the performed assessment of the situation and the prediction of its development influences the adoption of the decision to evacuate the population. After the decision has been adopted the choice of the optimum plan of evacuation with the help of the system is performed, then warning people is done and a special rescue group is informed about the evacuation through the modem, telephone and radio connections; sirens are switched on. Depending on the information from the facility, the chemical contamination sensors and the mobile lab, the actions connected with the evacuation are corrected. The time period from the moment the information about the occurrence of the emergency situation arrives, until the beginning of the warning should not be more than 15 - 20 min. A mathematical model of the PS cloud spread entered into the computerized system should produce the calculation of the movement of the front of the maximum-allowable and lethal PS concentrations. The data received from the chemical contamination sensors and from the mobile lab are necessary to carry out calculation corrections. The scheme of the work of such a system is given in Fig. 1.

Besides its main purpose, this system can be also very useful for training the facility personnel, and the services responsible for protection of the population and the personnel. It also can be used for training the population and the heads of the facilities and local authorities, whose responsibility it is to adopt the evacuation decision.

From the functioning of CW destruction facilities in Russia, another problem connected with the creation of up-dated security systems arises. However, because of the complicated economic situation, the works connected with the development of these systems are not performed. Mainly, only design works and the construction of the production infrastructure of the CW destruction plants is financed.

At the same time a number of foreign countries are planning to assist Russia in the field of CW disarmament. In the Udmurt Republic for the purpose of Lewisite destruction in Kambarka the Netherlands is going to invest 25 million guilders, Finland - 6 million marks, Sweden - 2,6 million kronas [10]; Italy and Great Britain expressed the intention to assist POS destruction in Kizner. One of the variants of such assistance can be participation in creation of the up-dated security systems in Kambarka and Kizner with the use of the equipment and modern computerized technologies that have already been created in these countries.

The Netherlands can offer the developments of "TNO Prins Maurits Laboratory". Sweden can participate in creation of the system of supervision and control over PS, and also in creation of the computerized system of planning the measures to be performed in the emergency case [11]. Finland can offer development of the companies TEKLA and ENVIRONICS to create the security systems.

CONCLUSIONS

The creation of up-dated security systems with the use of the modern PS analysis methods and modern computerized technology and means of communication is necessary for the successful functioning of the plant for CW destruction in Russia.

The creation of such systems can be realized at the expense of the international assistance offered by various countries to Russia in the sphere of CW disarmament.

KEYWORDS

Modern warning systems, emergency situations, chemical weapons (CW) storage buildings, CW storage sites, CW destruction facilities.

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FIGURES AND TABLES

Figure 1. The principal scheme of system operation

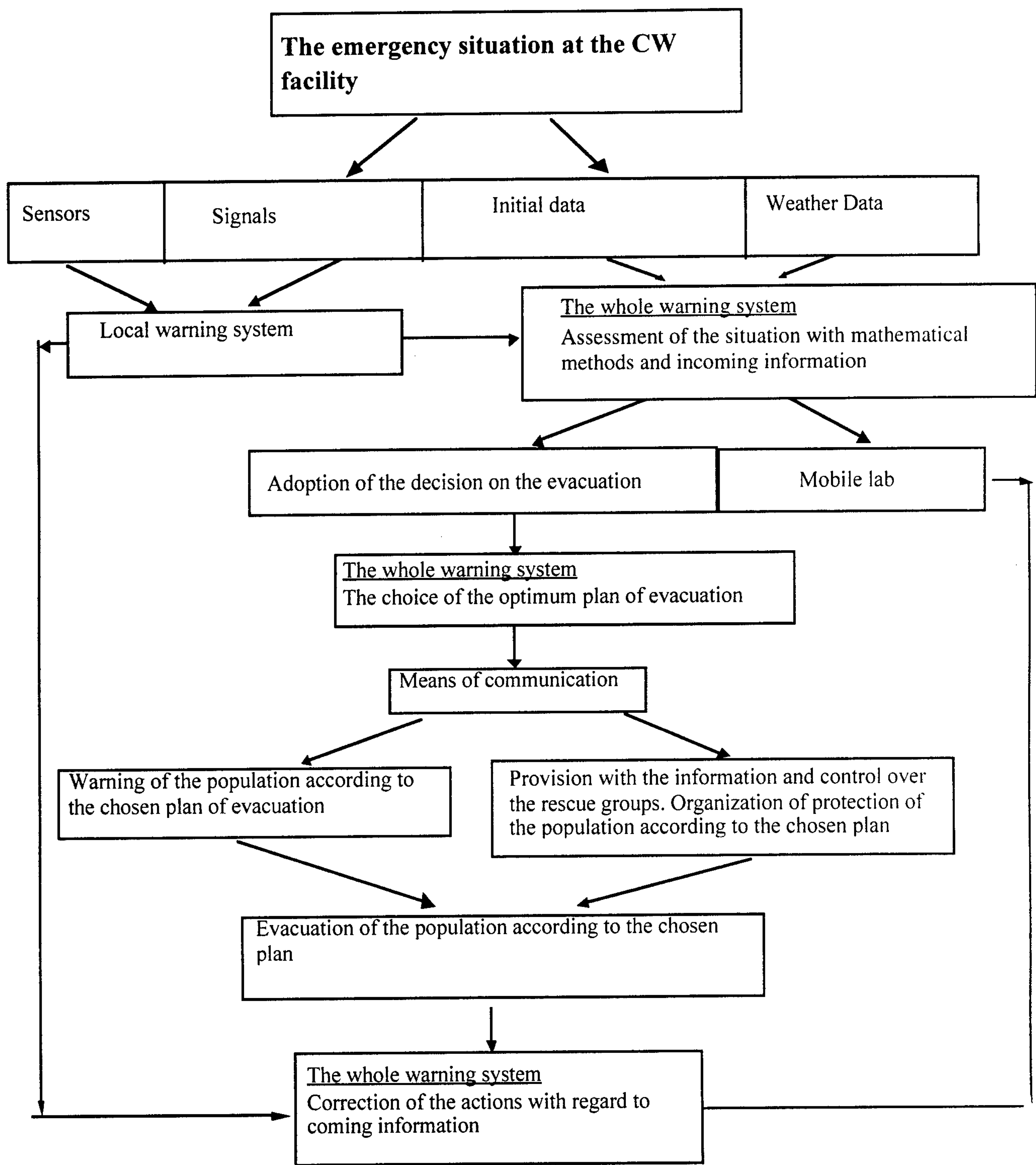

Table 1. The values of safe concentrations of PS in the atmosphere adopted in Russia and the USA, the levels of sensitivity of some devices and the time necessary for PS detection, mg/m^3.

Poisonous Substance	Safe Values of PS Concentrations		Devices			
	Russia	the USA	Russia			Finland
			GSP-11	GSA-12	KPHR-S	M-90
GB	$2 \cdot 10^{-7}$	$3 \cdot 10^{-6}$	$2 \cdot 10^{-3}$ - - $5 \cdot 10^{-2}$ (1-6 min)	$(6\text{-}8) \cdot 10^{-3}$ (4-7 min)	$5 \cdot 10^{-2}$ - - $2 \cdot 10^{-1}$ (1-30 sec)	$1 \cdot 10^{-2}$ - - $2 \cdot 10^{-2}$ (1-30 sec)
GD	$1 \cdot 10^{-7}$	$3 \cdot 10^{-6}$				
VX	$5 \cdot 10^{-8}$	$3 \cdot 10^{-6}$				
HD	$2 \cdot 10^{-6}$	$1 \cdot 10^{-4}$	-	-	-	-
L	$4 \cdot 10^{-6}$	$1 \cdot 10^{-3}$	-	-	-	-

50. VIRUS REPRODUCTION IN MACROPAHGES IS A COMMON FEATURE OF VIRAL HEMORRHAGIC FEVERS

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INTRODUCTION

Viral hemorrhagic fevers compose a group of 13 severe human diseases (table 1) with characteristic damage to hemostasis. The viruses belong to four families of enveloped RNA viruses: Arenaviridae, Flaviviridae, Bunyaviridae and Filoviridae. Causative agents of viral hemorrhagic fevers differ by morphology of the viral particles (Fig. 1), natural carriers and transmitters, clinical manifestations, and pathologic properties for humans and animals, and other biological characteristics. While many viral and bacterial infections may be associated with hemostatic impairment, hemorrhage is a hallmark of viral hemorrhagic fevers. Common pathological events are observed in all hemorrhagic fevers, such as thrombocytopenia, reduced levels of coagulation factors and vascular injury. Development of hemorrhages and disseminated intravascular coagulation is not very frequent, but severe complication indicating fatal outcome for many hemorrhagic fevers, including Rift Valley fever and Congo-Crimean fever, while such clinical manifestations are usual feature for filoviral Marburg and Ebola fevers [1]. It is interesting to analyze which pathogenic event may be common for viral hemorrhagic fevers.

MATERIALS AND METHODS

Studies of pathogenesis of filoviral hemorrhagic fevers are developing in SRC VB "Vector" more than 10 years. We examined Marburg and Ebola infections in monkeys and guinea pigs infected with various doses (from 1 LD_{50} up to 1000 LD_{50}) by intraperitoneal, aerosol and subcutaneous routes. Our main methodological approach was time course examination of the disease and application of small infectious doses. Animals were sacrificed daily during whole period of the infection. Samples for virologic, biochemical and microscopic studies were collected [2-5].

RESULTS AND DISCUSSIONS

Comparison of clinical signs and pathologic changes in Marburg and Ebola virus infected animals revealed many differences. Most evident of them were presence or absence of hemorrhages and signs of disseminated intravascular coagulation. Thus, guinea pigs infected with filoviruses never showed signs of hemorrhages or disseminated intravascular coagulation during clinical monitoring. Visceral organs of these animals also were devoid of morphological evidences for hemorrhages and fibrin thrombosis. Marburg virus infected green and rhesus monkeys developed hemorrhagic rash on the skin and mucous very rarely. Microscopic examination of monkey organs didn't show prominent hemorrhages and fibrin depositions. Bright differences in pattern of hemostatic impairment were observed in four monkey species infected with Ebola virus. Green and rhesus monkeys infected with the virus developed severe disease and died on days 6-8 after infection even with small doses. However, hemorrhages on skin and mucous membranes were detected very rarely. In contrast, about 50% of infected baboons demonstrated skin and mucous hemorrhages, as well as bleeding from nose, rectum and vagina. Microscopic studies found multiple hemorrhages in baboon visceral organs, while in green and rhesus monkeys fibrin thrombi and depositions were observed [3- 5].

Pronounced differences in damage to hemostasis in filovirus infected animals developing fatal infection gave a nudge that basic mechanisms of filoviral disease are lying in another organisms system. Time course examination of guinea pigs and monkeys infected with filoviruses established following common features of the infection:

virus replication in macrophages, hepatocytes, adrenal cortical cells, fibroblast and endothelial cells;
liver and kidney injury;
severe impact to immune system;
early and accrescent damage to microcirculation.

Ebola and Marburg viruses' replication in macrophages (Fig. 2) seemed first and main event, which determine the disease development. Macrophages provided primary filovirus replication and virus dissemination in organism. However, critical contribution of the macrophages in expansion of the disease was related to their functional activity. Virus infected macrophages released many biologically active substances, which influenced to hemostasis, vascular permeability, blood coagulation and immune defense. We believe that macrophage infection by filoviruses is the crucial pathogenic event for Marburg and Ebola hemorrhagic fevers development.

Macrophage system is the oldest regulatory system in living organism. These cells first were recognized as phagocytes providing defense of the organism against various invasions and dead cells. However subsequent investigations showed that macrophages are involved in many regulatory pathways. It is hardly to say which function of the organism may act without macrophage contribution. Macrophages conduct most vital events in organism – immune defense, blood clotting and vessel permeability and haemopoiesis. Our studies evidence for critical role of the macrophages in development of Marburg and Ebola hemorrhagic fevers. It is interesting to examine if the macrophages are involved in replication of another viruses causing hemorrhagic fevers.

Examination of the literature revealed various degrees of knowledge about macrophage significance for different viral hemorrhagic fevers. Many of them, for example, Kyasanur Forest disease and Omsk hemorrhagic fever, are described only in epidemiological and clinical aspects. However, ability to replicate in macrophages was shown for many viruses causing hemorrhagic fevers (Table 2). Postmortem examination of human organs revealed macrophages infected with Junin, Dengue, yellow fever, Lassa, Hantaan, Rift valley, Ebola and Marburg viruses. Unfortunately, no studies were performed establishing if the macrophages are first target cells for the viruses causing hemorrhagic fevers. This is not surprise because such studies need adequate animal models, which are not yet developed, and laborious time course investigations. It should be noted that filoviral hemorrhagic fevers may be modeled in monkeys with very close similarity to humans, and this is rather an exception for viral disease, then a rule.

Thus, replication in macrophages seems to be common feature for viruses causing hemorrhagic fevers. Another interesting data evidencing for macrophage significance for hemorrhagic fever development were published.

Correlation of the virus ability to replicate in isolated macrophages and to cause a disease in species donated macrophages, was established for Marburg virus. Examination of Marburg virus replication in peritoneal macrophages, isolated from monkeys, guinea pigs, rabbits and humans demonstrated a correlation of macrophage ability to reproduce Marburg virus in vitro and susceptibility of the organism for the virus [6]. Similar correlation was shown for Junin virus strains differing in pathogeneicity for rats [7, 8]. Replication of Junin virus in macrophages was shown to be a mechanism providing age-dependent resistance for the infection in natural host Calomys musculinus (this is a kind of rats) [9] and in white rats [10].

Dengue hemorrhagic fever is the most thoroughly investigated disease. Primarily infected humans demonstrate influenza-like disease, usually non-fatal. However, secondary infection lead to development of severe hemorrhagic fever very often associated with hemorrhagic shock and death. It was established that a mechanism of the shock development was based on macrophage infection by Dengue virus. Antibodies, circulating in the blood after primary infection exaggerated macrophage infection. In turn, infected macrophages released large amounts of biologically active substances, which induced hemorrhagic shock development. Dengue hemorrhagic fever and shock are evident example of the principal role of macrophages in hemostasis impairment [11].

The present data concerning pathogenesis of viral hemorrhagic fevers are not sufficient for making final conclusions about the role of macrophages in pathogenesis of these diseases. Meanwhile significance of macrophage infection is beyond all questions. Viral infection of the macrophages alter most vital systems, and damage of hemostasis is the most prominent and evident for hemorrhagic fevers. Undoubtedly, investigation of macrophage functions in viral hemorrhagic fevers will lead to development of new approaches for treatment of these dangerous diseases.

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KEYWORDS
Viral hemorrhagic fevers, macrophages, Ebola and Marburg infection

FIGURES AND TABLES

Figure. 1A. Marburg virus particles (Filoviridae family). .Negative staining

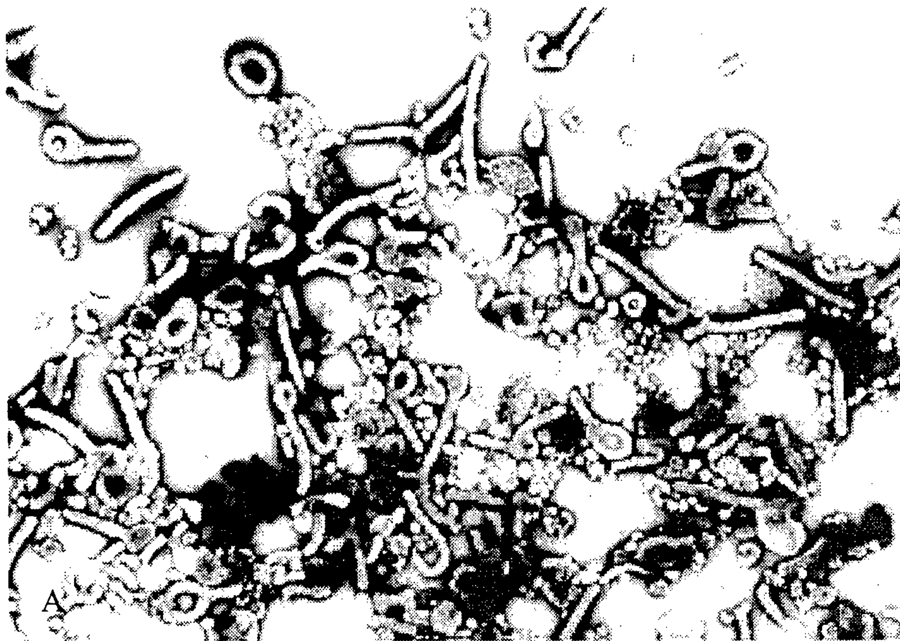


Figure 1B. 1 – Junin viral particles (Arenaviridae family); 2 – Congo-Crimean fever viral particles (Bunyaviridae); 3 – yellow fever viral particles. Negative staining.

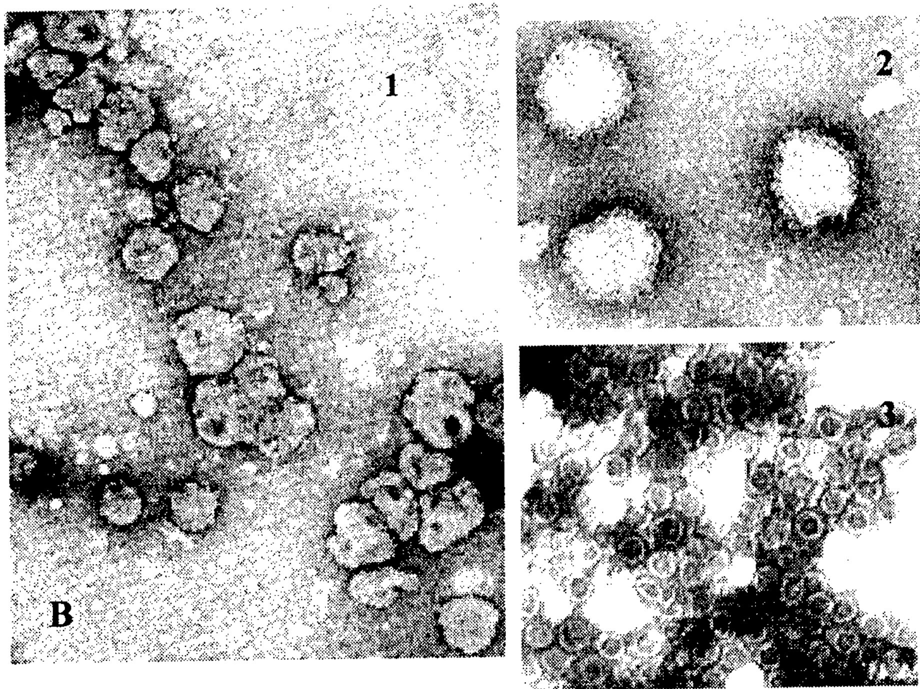
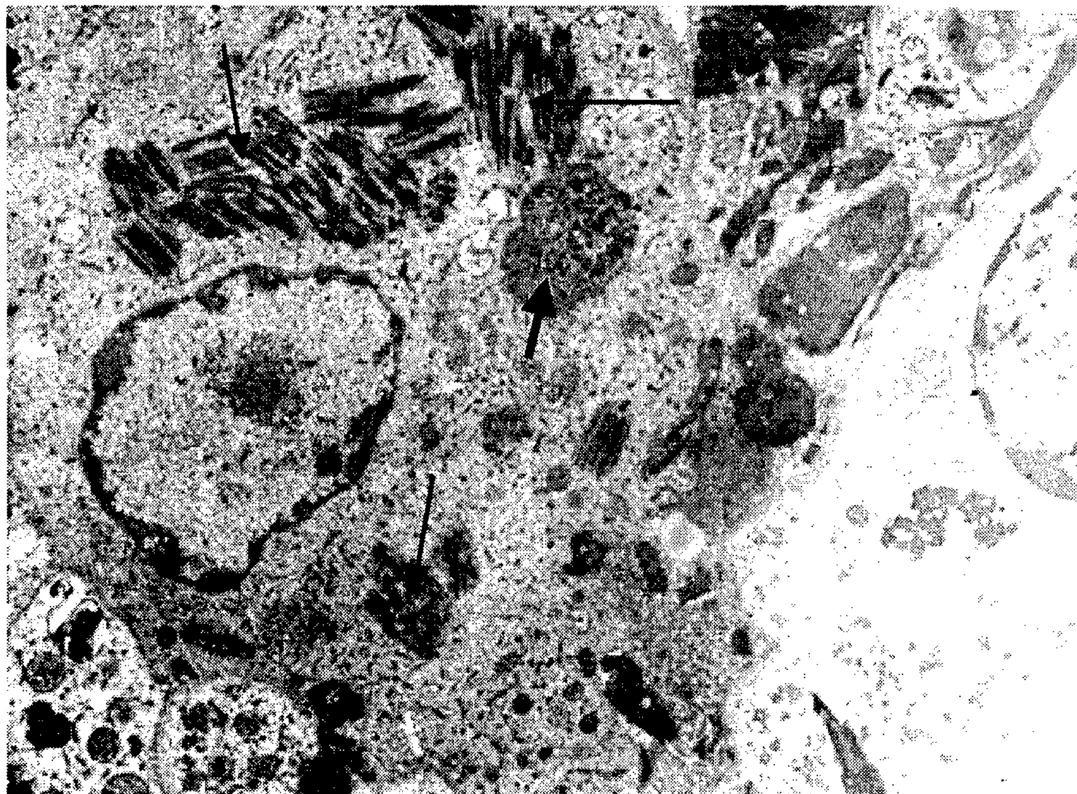


Figure 2. Ebola virus replication in macrophages. Arrows show viral inclusions in cytoplasm, thick arrows show phagosomes.



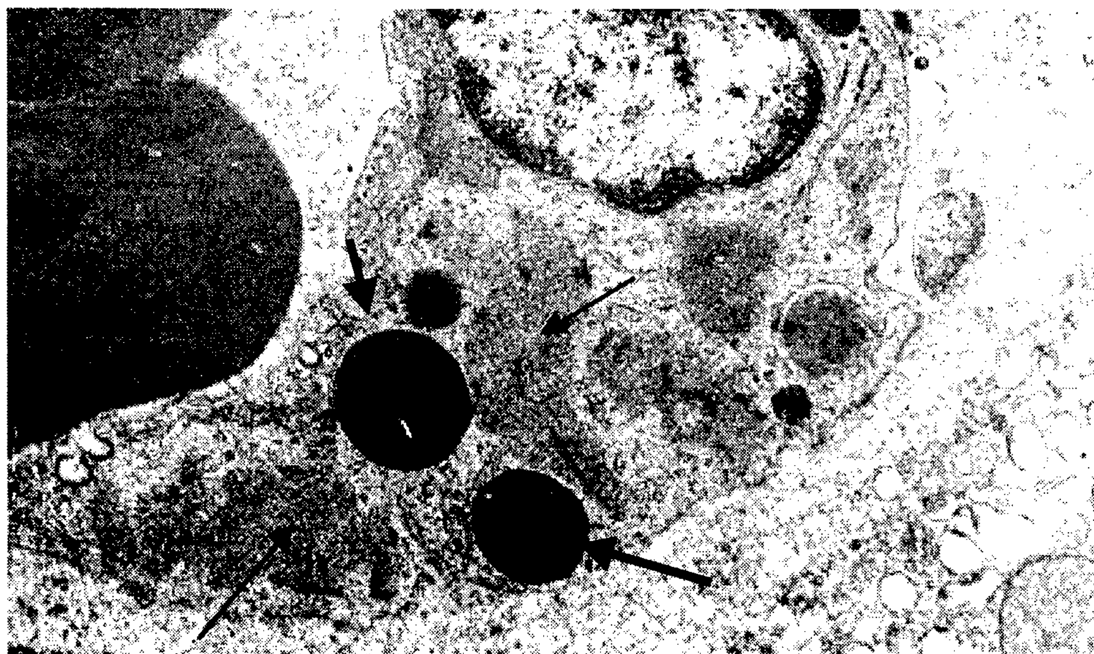


Table 1. Viral hemorrhagic fevers

Disease	Virus	Source of human infection
Argentine HF*	Junin	Rodent
Bolivian HF	Machupo	Rodent
Lassa HF	Lassa	Multimamate rat
Dengue HF	Dengue	Mosquito
Yellow HF	yellow fever	Mosquito
Omsk HF	Omsk HF	Tick (rodents, muskrats)
Kyasanur Forest disease	Kyasanur forest disease	Tick
Crimean-Congo HF	Crimean-Cingo HF	Tick
Rift Valley HF	Rift Valley	Mosquito
HF with renal syndrome	Hantaan	Rodent (striped field mice)
Marburg HF	Marburg	Unknown (bat?)
Ebola HF	Ebola	Unknown (bat, rodent?)
Venezuelan HF	Guanarito	Rodent

HF* - hemorrhagic fever

Table 2. Replication of viruses causing hemorrhagic fevers in macrophages

Virus	In humans	In animals
Junin	+ [12, 16]	+ [8-10]
Machupo		
Lassa	+ [17]	
Dengue	+ [11,13]	
yellow fever	+ [19]	
Omsk HF		
Kyasanur forest disease		
Crimean-Cingo HF		
Rift Valley	+ [20]	
Hantaan	+ [15]	
Marburg	+ [18]	+ [4]
Ebola	+ [18]	+ [4]
Guanarito		+ [14]

52. SCANNING FLOW CYTOMETRY FOR KINETICS STUDY OF ANTIGEN-ANTIBODY INTERACTION ON THE CELL SURFACE

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INTRODUCTION

Term "antigen" unites different pathogens (viral, bacterial etc.) to influence on a human organism. Antigen to antibody binding plays a crucial role in many biological processes. The new knowledge about this phenomenon will help us in better understanding of the complex processes like an immune response, a virus reproduction, which involve binding of antigen as first stage (1). On other hand, preventive measures and quantitative characterisation of different antigens are necessary to predict influence of these antigens on organism. Also quantitative assessment of the resistance of cells and ways to change this resistance are necessary. The fast assessment of the resistance of cells seems to be particular interesting and important for new or dangerous pathogens.

So far as all processes in an organism develop in time, our knowledge should concerns time dependencies of the antigen interactions with cells. Also, kinetic study of these processes is more preferable than equilibrium study because instead only affinity constants both forward and forward rate constants could be observed. Moreover, mathematical modelling of these processes give a researcher a new ways to characterise a cell population.

The development of new experimental instrumentation has permitted the real-time analyses of ligand-receptor binding. One of them is a flow cytomety. Flow cytometry allows the direct measurements of antibody binding to cell receptors on individual cell (2). Here we present both instrumental and mathematical approaches of the flow cytometry – Scanning Flow Cytometry (3) for the kinetic study of the antigen-antibody interactions on the cell surface. Kinetic experiments of the binding of anti-mouse immunoglobulins to the murine cells were performed to demonstrate applicability and potential of the suggested approaches.

MATERIALS AND METHODS

Antibodies and cells.

Murine hybridomas 7H10 and 9G6 (3) bearing IgG-like receptors were used as antigen-presenting cells. The cells were washed three times and resuspended in the buffered saline (0.01 M phosphate buffer, pH 7.2 with 0.15M NaCl). Then the cells were stored at 4^0C and used within a working day. Affinity purified rabbit anti-mouse immunoglobulins IgG (Sigma) labeled with dye were used as antibodies against hybridoma cells in these experiments. Immunoglobulins were labeled by fluorescein isothiocyanate molecules (FITC) prior experiments. FITC-labeled immunoglobulins were freeze-dried and stored at 4^0C. The FITC-conjugates were dissolved in buffered saline immediately before experiments. The number of FITC molecules conjugated per single IgG molecule was determined with a spectrophotometer according to Storz (4).

Kinetic study of the antibody binding to cells.

All kinetic experiments were carried out at room temperature 25^0C. FITC-conjugates dissolved in buffered saline were mixed with the cells and than incubated during 1 hour in the plastic tube that was attached to the inlet capillary of the Scanning Flow Cytometer. Single cells from mixture, one by one, flew through the Scanning Flow Cytometer during the hour. Light scattering pattern and fluorescence were measured simultaneously for each cell. Light scattering signal was used for cell identification. The fluorescence signal obtained from the cell is proportional to the number of immunoglobulins molecules attached to cell surface.

Scanning Flow Cytometry and fluorescent immunoassay.

Flow cytometry is one of the powerful techniques in the detection and characterization of the individual cells and particles with high rate. The Scanning Flow Cytometry (SFC) is a new approach expanding possibilities in this area. SFC concept differs from conventional flow cytometry in two essential points (3): 1) the laser beam is coaxial with the flow and 2) the optical system allows measurements of the entire angular light-scattering pattern, an indicatrix (Fig.1). To be sure, the indicatrix contains an information about the cell size and refractive index, cell structure and shape. It is obviously, that entire light scattering pattern provides more information about the cell than ordinary wide angle, forward and side scattering concepts. Each cell moving through the scanning optical cuvette gives a light scattering signal depended on time. This experimental signal is so called native SFC trace (6). SFC traces are unique for each cell and content a great potential for particle classification (7). Native SFC records (traces) have been used for cell identification in the experiments presented here. One native SFC trace given up by a latex particle is shown

in Fig.2a. A native SFC trace could be transformed to the angular dependence of the light scattering (indixcatrix). But we use the native SFC traces to identify cells among irrelevant particles, such as cell clumps, damaged or dead cells, and protein aggregates in these experiments.

Interesting as light scattering is in itself, SFC is combined with fluorescent immunoassay in work presented here. So, fluorescence excited by Ar-laser (Spectra Physics, 15-mW, 488 nm) could be measured simultaneously with light scatter. Thus equipped SFC becomes a powerful and useful tool for study antigen-antibody interactions on intact living cells. An example of conventional experimental signal from one particle is shown in Fig.2. Up curve is light scattering signal, namely native SFC trace. Bottom curve is signal from fluorescent channel. Peak corresponds fluorescence from the particle. Area of fluorescent peak is proportional to the number of dyes molecules attached to the particle.

Mathematical model.

As mentioned above, quantitative characterization of the interaction between antibodies and their aim on the cell surface are necessary to predict the action of the antigens in organism. On other hands, flow cytometry measurements result in a huge block of numerical data, a so mathematical interpretation is necessary to derive values from experimental results. . To obtain numerical values from fluorescent experimental data a mathematical model of interactions between antigens and antibodies has been suggested. Usually mathematical models describe only the mean values (8, 9). But flow cytometry gives more information for researcher, namely a cell distribution on cell receptors. So, for better understanding, distribution of cells should be included in the mathematical model. The suggested model bases on the distribution of cells. Main assumptions can be formulated in follows manner. Antibody to antigen (receptor) binding concern as bimolecular chemical reaction. It means that no conformation changes are supposed. Also, exo- and endocytosis are not included in the mathematical model. Accurate mathematical formulation results in a set of the differential equations. Analytical solution of the set could be derived both for reversible and irreversible binding of antibodies to cell receptors. This solution describes time evolution of cell distribution by bound and unbound receptors. Hence, if one will know the initial distribution on cell receptors. rate constants, one can predict how the process of antibodies binding to cell will be going. Moreover the model enables to characterise examined pair of antigen and antibody by kinetic constants numerically.

RESULTS AND DISCUSSION

In order to demonstrate the applicability of the presented approaches both instrumental and mathematical, kinetic experiments of mouse-antibodies to murine hybridoma cells binding were carried out with the Scanning Flow Cytometer. As mentioned in previous chapter, the native SFC light-scattering traces have been used for cell discrimination among irrelevant particles. One can estimate of potential of the SFC technique for cell identification and discrimination from fig.2b. Experimental SFC traces from different single cells measured in preliminary experiments are shown in this figure. There are murine hybridoma, human sarcoma, and two bacteria *E.coli* from different strains. This picture illustrates differences in the light scattering patterns for different species of the cells. It is obviously, that new possibilities in micro-organism detection and characterisation arise. One of the ways of application SFC technique in this field consists in creation of the database of light scattering properties of different micro-organism species.

For every valid cell determined by light scattering, the integral of the fluorescence signal was evaluated. The fluorescence signal obtained from the cell is proportional to the number of immunoglobulins molecules bound to the cell. So kinetics of the antigen-antibodies interaction has been measured directly in real time.

The fluorescence signals of 500 cells were collected to build the fluorescent intensity distribution of cells. The kinetics of cell fluorescence intensity histogram for 7H10 is presented in Fig.3. As it follows from experimental data, the whole reaction takes approximately 1 hour. The kinetics of cell fluorescence distribution was fitted with suggested mathematical model. The solid lines shown in Fig.2 are the best fit to the experimental data. The parameters obtained from the fitting and reaction conditions are presented in Table 1. There are presented the known parameters of the system – initial concentrations of the antibodies ($A(0)$) and cells (C) . All other parameters in the Table 1 were obtained from the fitting, i.e. the ratio between initial concentration of the cell receptors ($I(0)$) and initial concentrations of the antibodies $I(0)/A(0)$, the time of the reaction $(k_f(A(0)-I(0)))^{-1}$, the association rate constant k_f of a single binding site, and the average amount $\bar{n}$ of binding sites on a cell. It is to be noted that estimation of the number of collisions between a dissolved imminoglobulin and a cell receptor required creating an antigen-antibodies complex is possible from mathematical model. An antigen antibody complex was formed in our experiments after approximately 200 collisions. This value is relevant for used pair of the anti-mouse IgG immunoglobulins and murine hybridomas and can vary for another antigen-atibody pair. Also fitting results show

differences between the two clones in averaged number of cell receptors on one cell (n) and forward rate constants k_f. Values of the n and k_f obtained in different initial conditions are in good agreement for one clone 9G6 but differ more sharply for different clones 9G6 and 7H10.

The most important result of the model application is that the initial cell distribution on free receptors (binding sites) is obtained from the experimental data with the mathematical model. Fig.4 shows the result of the reconstructing the initial distribution on cell receptor from the experimental data for 7H10 cells by the mathematical model. The initial cell distribution obtained from the fitting coincides with the final experimental cell distribution on occupied receptors. Such coincidence indicates that there is the high affinity constant of the binding, and, therefore, nearly all cell receptors is occupied by ligand at the end of the reaction. For the low binding affinity there are no such coincidence between the distributions, and in that case the mathematical model may present the only way to find the cell distribution on free receptors.

Following the concept of distribution function approach, we have presented a mathematical model of the binding, which allows us to obtain the kinetic parameters of the reaction and to find the initial cell distribution on free binding sites. This may become of particular interest for informative statistical characterization of cell populations, and for detailed quantitative description of immune reactions in cell systems (10). Thus, we believe that the current approach is helpful for the understanding of antibody to antigen binding in cell systems and thus for assaying and designing cell populations.

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KEY WORDS

Flow cytometry, light scattering, characterisation, identification, mathematical model.

FIGURES AND TABLES

Figure 1. Scheme of the Scanning Flow Cytometer.

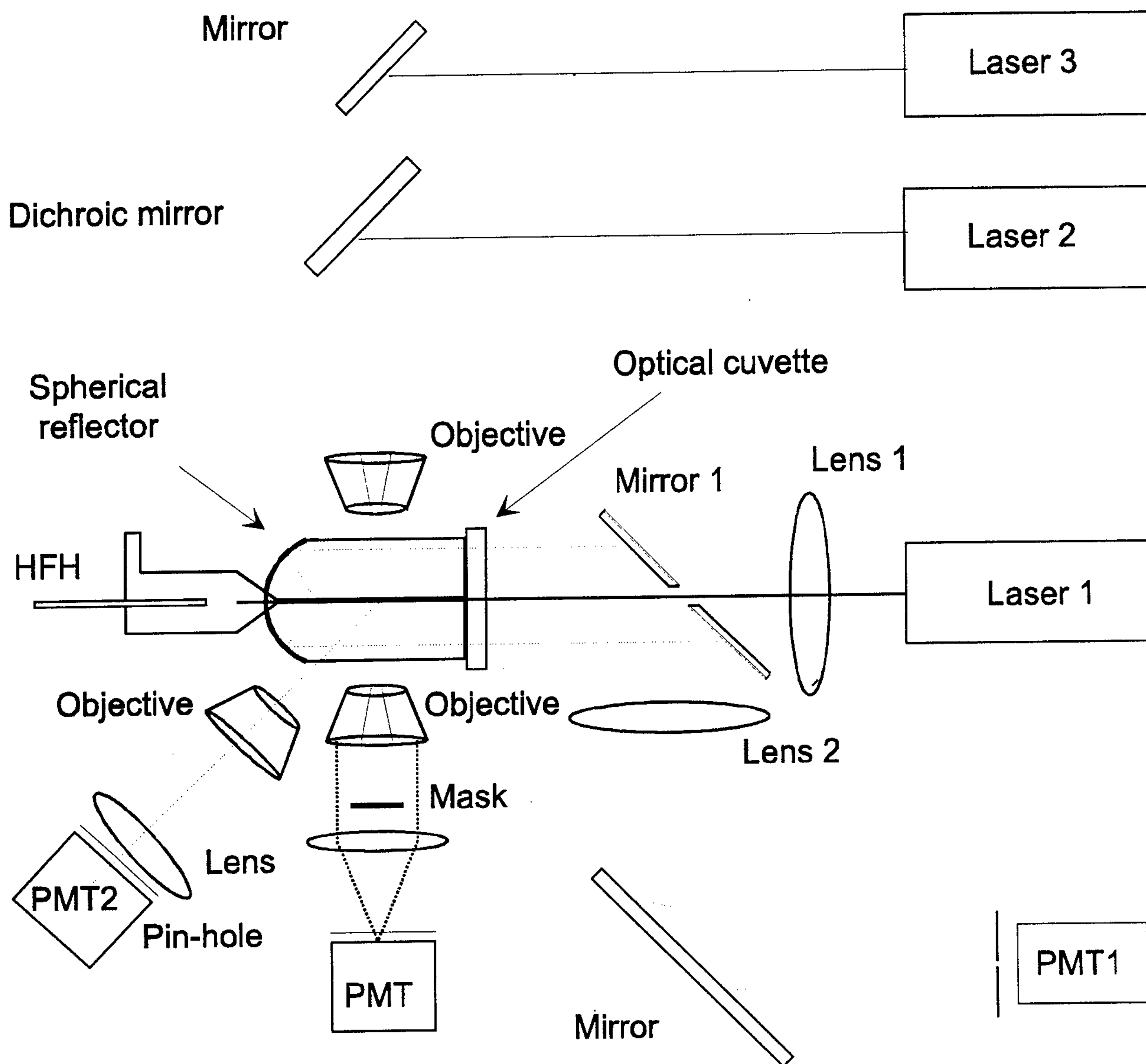
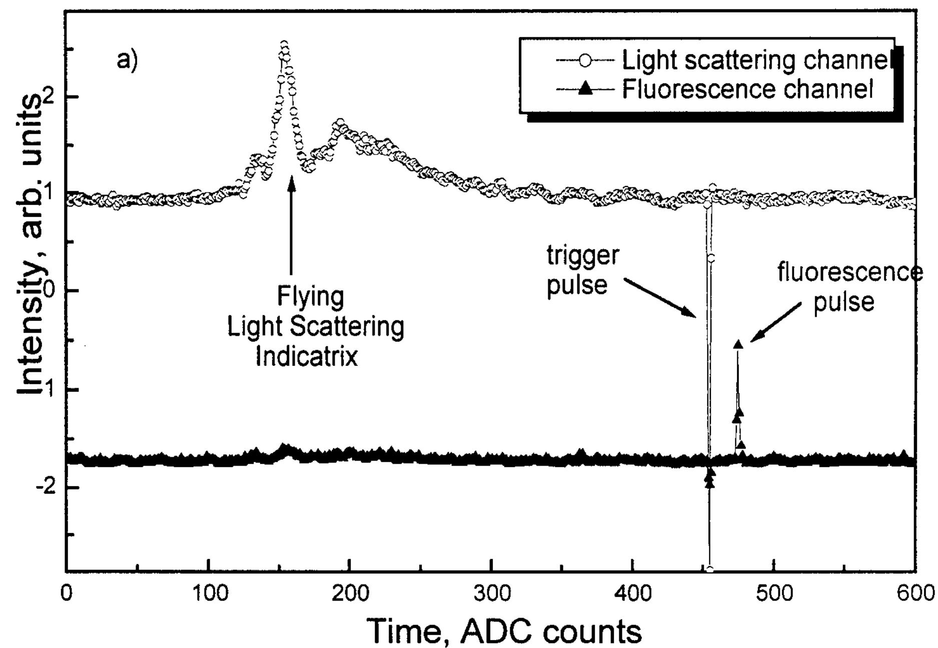


Figure 2. Examples of the experimental signal from Scanning Flow Cytometry.



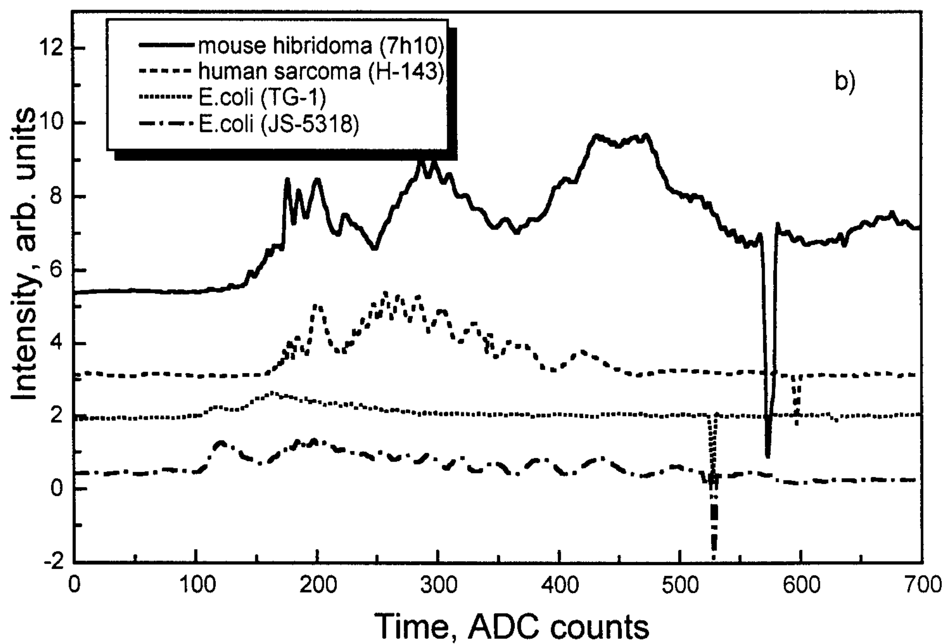


Figure 3. Time profile of fluorescence intensity histogram for 7H10 cells incubated with FITC-labeled IgG immunoglobulins. The points and solid lines correspond to experimental data and theoretical calculation, respectively.

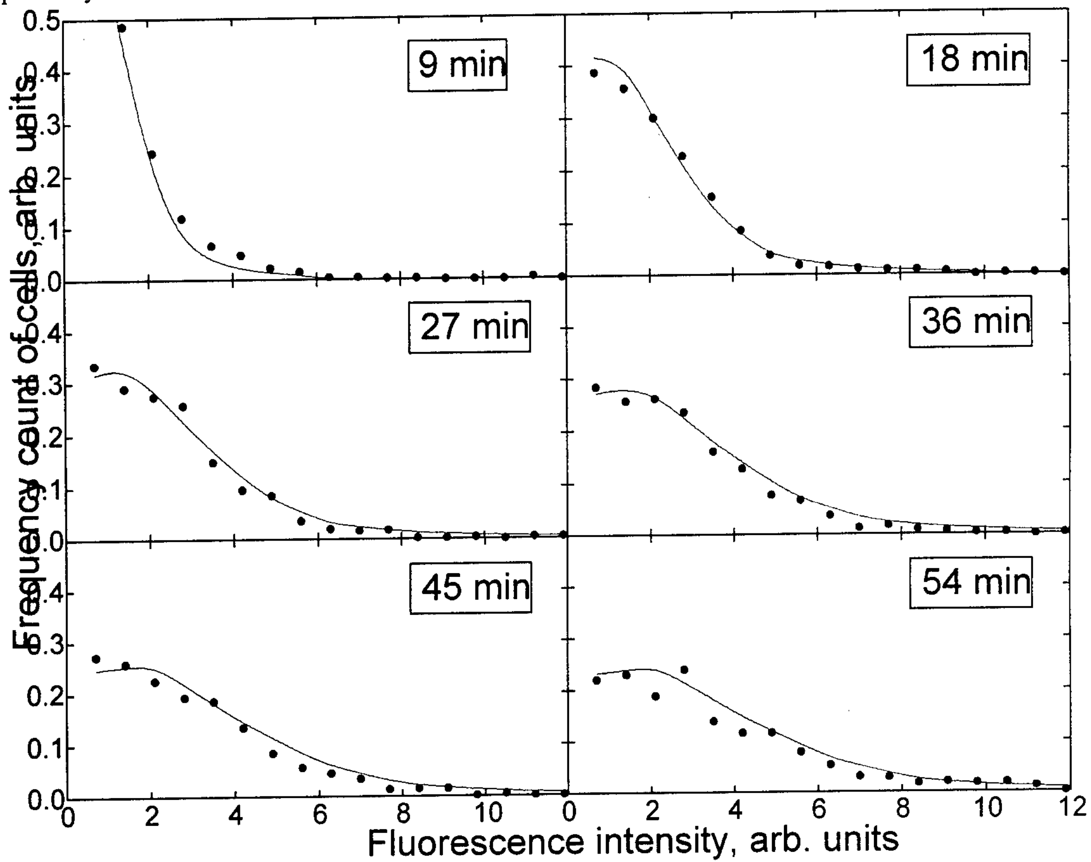


Figure 4. Initial distribution on free binding sites for 7H10 cells reconstructed from the experimental data using the mathematical model

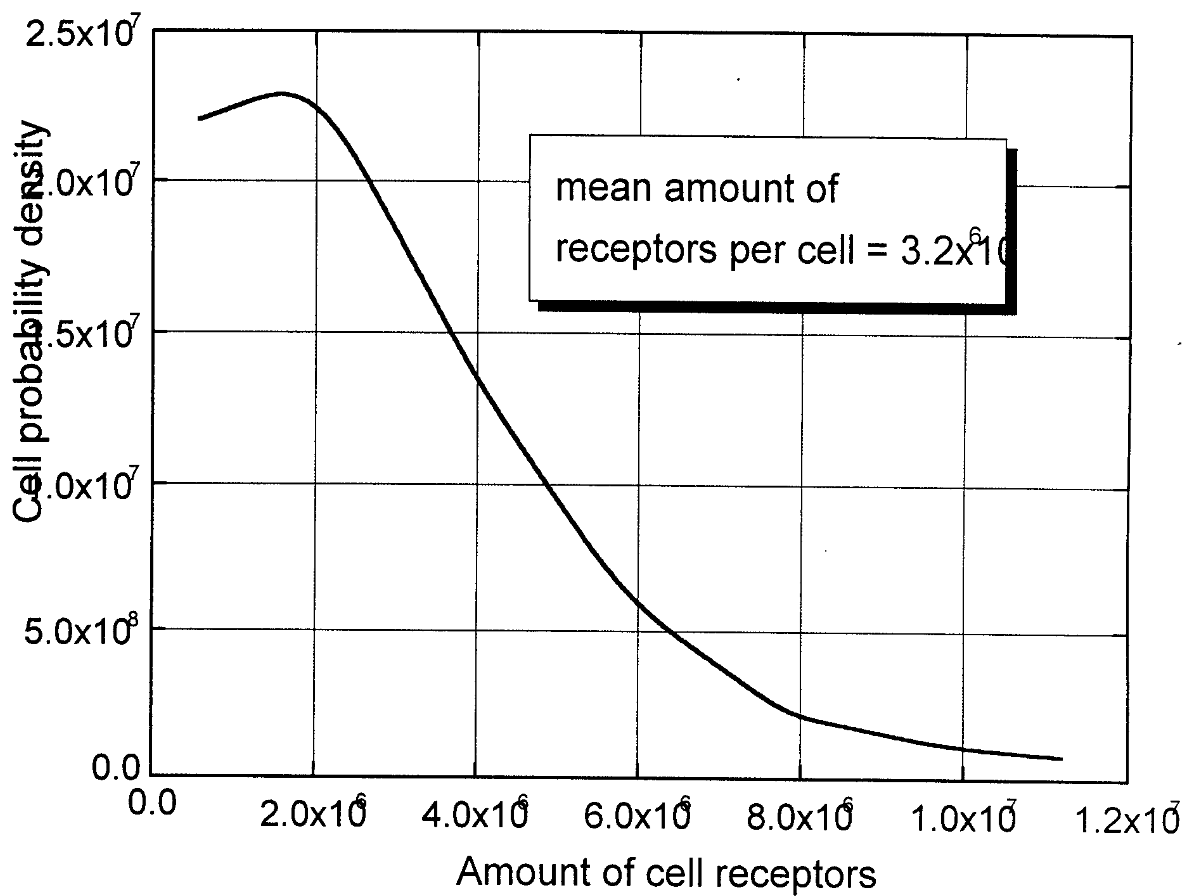


Table 1. Conditions and fitting results for ligand-receptor binding.

Parameter	Sample 9G6_b	Sample 9G6_c	Sample 7H10_a
$A(0)$, cm^{-3}	3.1_10^{13}	6.3_10^{13}	1.3_10^{14}
C, cm^{-3}	1.0_10^{6}	1.0_10^{6}	2.0_10^{7}
$I(0)/A(0)$	0.031_0.009	0.016_0.006	0.49_0.13
$(k_f(A(0)-I(0)))^{-1}$, s	502_124	290_76	607_84
k_f, cm^3s^{-1}	(6.3_1.6)_10^{-17}	(5.4_1.4)_10^{-17}	(2.5_0.4)_10^{-17}
n	9.6_10^{5}	10.1_10^{5}	3.2_10^{6}

53. STRUCTURE AND EFFICIENCY OF CARBAMATES AS DRUGS FOR PROPHYLAXIS

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The usual treatment for organophosphate (OP) poisonings is a combination of atropine and some oximes. This is not effective against poisoning by soman, cyclosarin and tabun on account of rapid dealkylation of acetylcholinesterase (AChE). Previously, it has been shown that pretreatment with certain cholinesterase reversible inhibitors like physostigmine gave appreciable protection against the poisoning by any OP, including soman. In this paper a number of carbamates have been tested for their ability to protect mice, rats, cats and dogs against paraoxon, armine and soman poisoning.

A series of phenyl, naphthyl and pyridyl carbamates with different chains between rings and with different substituents on the rings were tested. In the experiments in vitro the anticholinesterase activity of carbamates and the influence of compounds on phosphorylation of AChE by OP was studied also. There was no correlation between the protective activity of the carbamates and either their chemical structure or toxicity and anticholinesterase activity.

The present study has shown that protective effect of carbamates no doubt depends primarily upon the ability of the carbamate to inhibit the brain AChE with the partial protection of the enzyme against following phosphorylation. Many factors (such as their ability to cross the blood - brain barrier, duration of anticholinesterase action, inhibition of brain AChE reversibly, ability of acetylcholine to increase the speed of gradual decarbamylation of enzyme) are involved in determining of protective efficiency. It has been found that some carbamates with irreversible mode of action increased the toxicity of OP. The efficiency of carbamates also depends on animal species. In mice and rats carbamates gave a weak protection whereas in cats and dogs the protective effect was significantly more. A few most effective carbamates with a longer duration of protective action than physostigmine were study in more detail in conjunction with atropine and as a components of prophylactic antidotes.

Keywords: carbamates, structure, activity, organophosphates, prophylaxis

55. BIODEFENSE VACCINES: REGULATORY & MANUFACTURING ISSUES AND CONSTRAINTS

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ABSTRACT

Vaccines like other medicinal products are subject to strict regulatory controls designed to protect recipients from products that are unsafe and/or ineffective. To gain regulatory approval manufacturers must also demonstrate that the conditions of manufacture ensure product quality and consistency whenever a batch is produced.

Most potential biological warfare agents do not naturally produce human disease on a scale that allows epidemiological clinical trials that are the usual basis for determining efficacy. Deliberate human challenge is unethical and contrary to the Nuremberg Code. Accordingly, efficacy determination is reliant upon a combination of animal challenge and immunogenicity studies, coupled with immunogenicity studies in humans. For several of the diseases the protective immune response is complex involving both T and B cell responses. Identifying surrogate markers of efficacy is therefore a challenging, but nonetheless essential requirement.

Biodefense vaccines have for a long time been the "orphans" of vaccinology. Relative to traditional public health vaccines they are not required in large quantities. Even with the renewed interest in this class of products it is very unlikely that it will be economically feasible to have production facilities dedicated to each product. Multiple use of facilities, especially if production of any one vaccine is infrequent, poses further challenges.

It is salutary to note that fully licensed vaccines are currently approved for only two biowarfare agents. To meet the need for biodefense vaccines and to achieve the high standards that are essential will require specialized producers having close working relationships with government R&D facilities and regulators. Investment by Governments to create and sustain such capabilities is essential.

57. SWISS CONTRIBUTIONS TO THE IMPLEMENTATION OF ARTICLE X OF THE CHEMICAL WEAPONS CONVENTION (CWC)

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INTRODUCTION

Article X of the CWC clearly shows the importance and value of creating a viable and meaningful assistance package in case States Parties are victims of CW use or threat of use. It discourages potential aggressors to benefit from the elimination of CW; contributes to the universality of the CWC and gives incentives to States to give up chemical weapons option.

From Swiss point of view, concrete pledges of assistance are prerequisite for a successful and credible implementation of Article X. Upon request by a CWC State Party, Switzerland will make available specialists from the Swiss NC Defense Division, NBC individual and collective protection equipment and expertise needed for the training.

Furthermore, Switzerland is prepared to supply individual and collective means of protection, decontamination, and detection.

CHIEF INSTRUCTOR TRAINING PROGRAM COURSES (CITPRO)

Switzerland was one of the first countries that made a concrete offer of assistance under Article X.

An important element of this of this offer was a CW protection-training program. The main purpose of this Chief Instructor Training Program (CITPRO) was to train specialist and experts from State Parties who are, or will be associated with CW protection and training of civilians in their home countries. In more concrete terms, CITPRO was designed to assist other CWC Member States in their efforts to establish a basic CW protection capability that will directly benefit the civilian population and to familiarize States Parties with Swiss offered protection and decontamination equipment.

The Government of Switzerland and the Organization for the Prohibition of Chemical Weapons (OPCW) Technical Secretariat jointly organized the two CITPRO Courses.

CITPRO-COURSE I/1998 (23-27 November 1998)

Forty Chief Instructors representing 30 States Parties along with 2 participants from the Secretariat attended this course, which took place at the NC Training Center in Spiez. The participating States Parties were Argentina, Brazil, Bulgaria, Burkina Faso, Cameroon, Chile, Cuba, Czech Republic, Ecuador, Ethiopia, India, Iran (Islamic Republic of), Italy, Jordan, Kenya, Kuwait, Latvia, Lithuania, Malta, Mongolia, Oman, Pakistan, Peru, Romania, Slovak Republic, South Africa, Sri Lanka, Sweden and Turkey.

CITPRO-COURSE II/1999 (25-30 April 1999)

55 Chief Instructors representing 32 States Parties along with 2 participants from the Secretariat attended this course, which took place at the NC Training Center in Spiez. The participating States Parties were Argentina, Belarus, Belgium, Bosnia and Herzegovina, Brazil, Chile, China, Croatia, Ethiopia, Ghana, India, Indonesia, Iran (Islamic Republic of), Korea, Latvia, Mauritius, Moldova, Morocco, Pakistan, Paraguay, Peru, Philippines, Poland, Romania, Slovenia, Sri Lanka, St. Lucia, Sweden, Tunisia, Turkey, Venezuela and Zimbabwe.

CITPRO-COURSE III/2000 (2-7 April 2000)

35 Chief Instructors representing 33 States Parties attended this course, which took place at the NC Training Center in Spiez. The participating States Parties were Algeria, Argentina, Armenia, Bolivia, Brazil, Burundi, China, Croatia, Cook Islands, Ecuador, Fiji Islands, Indonesia, Italy, Jordan, Kenya, Latvia, Lithuania, Mali, Malta, Mauritius, Mongolia (2), Morocco, Namibia, Pakistan (2), Peru, Philippines, Poland, Romania, Sri Lanka, Sudan, Ukraine, Uzbekistan.

ORGANIZATION OF CITPRO COURSES I-III

After an introduction by official representative of the Swiss government and of the OPCW, the course began with general lectures on "Chemical Threat Types and Effects of CW" and on "CW prevention, protection and decontamination". Afterwards the participants were divided into four working groups for practical work and each of them received his/her individual protective equipment, which was used during the whole course.

The first practical course taught by Swiss Armed Forces Instructors was on CW respiratory protection and protective mask. On the second and third day the practical courses centered on skin protection and CW protective clothing. On the theoretical part, presentations were also given on the second day on the coordination of international rescue and on medication-prophylactic and therapy procedures. The general presentations on the third day focused on CW risk prevention, emergency and long term measures and on domestic alarm systems.

The practical work on the fourth day centered on CW decontamination: handling equipment and procedures while the theoretical aspects were made on monitoring and detection of CW agents and an industrial chemical accidents. On the fifth and the last day of the course, exercises on multi-task training and testing methods were carried out. Participants were also given the opportunity to present their questions and problems raised during the course. In the afternoon, the participants visited the Spiez NC Laboratory for familiarization with detection and methods of dealing with actual CW agents. At the end of the course individual protective equipment provided to each participant for practice was finally given to the participants as a gift of the Swiss Government.

SWISS EMERGENCY FIELD LABORATORY (SEF-LAB) TRAINING COURSES

The Government of Switzerland and the Technical Secretariat of the OPCW jointly organized the courses, which were the first of this kind.

The courses provide training in civil CW detection for up to 16 chief instructors from Member States of the OPCW. SEF-LAB provide basic training for specialists who are (or will be) associated with CW detection training in their home countries.

Its main purpose is to assist other OPCW Member States in their efforts to establish a basic CW detection capability that will directly benefit the civilian population. The training will enable participants to properly use the detection equipment contained in the Swiss pledge pursuant to Article X.

The course draws on the extensive experience of the Laboratory specialists of the Armed Forces in the field of technical and personnel support to the civilian authorities. These specialists are equipped with CW detection-equipment (Thin Layer Chromatography) for a simple but reliable qualitative and semi-quantitative detection of chemical agents in air, soil, water and food in case of CW disaster.

The use of this equipment provides information under aggravated circumstances without power supply.

Due to the objective of the course and its technical nature, the candidates are carefully selected. Applicants from Member States, which do not yet have civilian CW detection and analysis capabilities, are particularly welcome. Participants should have a basic technical (laboratory) and chemical background.

SEF-LAB COURSE I/1999 (14-19 November 1999)

16 participants representing 16 States Parties attended this course, which took place at the NC Training Center in Spiez. The participating States Parties were Algeria, Argentina, Bangladesh, Brazil, China, Ethiopia, Iran, Italy, Kenya, Lithuania, Peru, Romania, Saudi Arabia, Slovenia, Vietnam, Zimbabwe.

SEF-LAB COURSE II/2000 (14-19 May 2000)

15 participants representing 15 States Parties attended this course, which took place at the NC Training Center in Spiez. The participating States Parties were Algeria, Argentina, Bulgaria, Cuba, El Salvador, Ethiopia, Fiji Islands, Jordan, Kuwait, Morocco, Namibia, Romania, Slovak Republic, Sudan, Zimbabwe.

58. COMPILATION OF A TRAINING CURRICULUM FOR CB DEFENSE SPECIALIST ADVISERS FOR THE SOUTH AFRICAN NATIONAL DEFENSE FORCE

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ABSTRACT
In recent years, religious cults and other fanatical groups worldwide have increasingly resorted to CB weapons to achieve their goals. Furthermore, the alleged possession of the technological requirements to produce CB weapons, combined with the suspected intent to use such weapons by a number of North African and Middle Eastern countries who are non-state parties to both the Chemical and the Biological and Toxin Weapons Conventions, has raised considerable international concern over the possible use of CB weapons, particularly in unconventional scenarios. In line with the global awareness of this threat and the inescapable requirement to maintain and continuously develop effective CB defensive countermeasures, the compilation of a training curriculum for specialist advisers on these aspects has become an imperative component of South Africa's overall defense strategy.

As the recognized primary South African CB defense R&D institution, Protechnik Laboratories was tasked with this assignment in collaboration with personnel from the South African Military Health Services (SAMHS) and other stakeholders. The curriculum is aimed at candidates who will serve on an advisory basis at unit and formation levels in all arms of service of the SANDF. Consequently, a balanced approach, focusing on the essential theoretical background, but without neglecting practical aspects of CB defense, had to be adopted. This in turn, required different areas and levels of emphasis in respect of course content and depth of knowledge that are deemed essential for such candidates to fulfill their mission.

A brief outline of the curriculum and an overview of the first course (presented during October 1999) is presented.

INTRODUCTION
Chemical defense training in South Africa dates back to shortly after WW II. At that time, limited training programs were conducted by the SA Army College and the NBCD School in Simonstown, who made use of manuals and related information based on British doctrines dating from WW II. During the ensuing years, the chemical threat existed and expanded between NATO and Warsaw Pact countries, but South Africa, being far removed from that sphere, did not then consider chemical defense to be a strategic necessity. During the South West African border conflict of the early 80's however, the then SADF became aware of the possible use of chemical weapons against South African forces, and since then, a comprehensive chemical defense program was embarked upon. This included the development of training programs and courses, which were aimed at protection and survival of personnel, and the maintenance of operational efficiency under attack by chemical weapons. In broad terms, these courses covered the classical areas of detection, protection and decontamination, while a separate course was instituted for medical countermeasures such as prophylaxis and treatment. A detailed discussion of these early developments is beyond the scope of this presentation, and will not be further addressed here.

International developments during recent years, such as the entry into force of the Chemical Weapons Convention, the Tokyo subway incident of 1995, chemical weapons proliferation in Iraq and North African countries, and closer to home, continued political instability and unrest in Lesotho, the Democratic Republic of the Congo, Angola and Zimbabwe necessitated a reassessment of South Africa's chemical defense strategy and capabilities. Against this background, it stands to reason that updated and dedicated training programs, and in particular, a training course for specialist advisers on chemical and biological defense, are essential components of an overall defense strategy from a South African viewpoint.

This contribution is intended to elicit inputs and discussions from the floor, which might enable the further improvement of the proposed training curriculum.

COMPILATION OF A CB DEFENSE SPECIALIST ADVISERS TRAINING CURRICULUM
As a starting point, it was necessary to establish the status of CB defense training in South Africa by reference to training programs and curricula currently in use, followed by interactive discussions with various officers and instructors from the South African Military Health Services (SAMHS) and the South African Army and other stakeholders. Without naming them, their individual contributions are acknowledged here. Cognizance was also

taken of similar programs and documentation that could be obtained from international sources. In particular, the "Chemical and Biological Defense Handbook" (RMCS Green Book), 1996 edition, edited by K. Clark from the Royal Military College of Science at Cranfield University in Shrivenham in the UK, was used as a reference.

The following considerations were used as guidelines in the compilation of the training curriculum and the presentation of lectures:

The course duration would be 4 weeks (i.e. 20 days) with 8 lecture periods of 40 min each per day, amounting to 160 available lecture periods in total;

1. All candidates would have at least a Grade 12 (High School) qualification, and would preferably have successfully completed an instructor's course;
2. Trained candidates would serve on an advisory rather than an operational basis at unit and formation levels in all arms of service of the SANDF;
3. Emphasis would be on theoretical and practical training aimed at operational staff competency rather than on technical execution;
4. Practical demonstrations would be limited to the extent to which such demonstrations could facilitate the assimilation of lecture material;
5. Biological agents and defense would not be covered separately, but should be integrated in the different sections of the curriculum;
6. The curriculum would be subject to annual revision, which would provide for the inclusion of civilian disaster management and response, and level of SANDF involvement in such issues, at a later stage;
7. Time constraints would necessitate a substantial amount of self-study by candidates on certain aspects not particularly covered in the different sections of the curriculum.

The curriculum was divided into 12 main sections, each of which was further subdivided into a number of generic sub-sections, as outlined below:

MAJOR TOPICS AND SUBSECTIONS

1. INTRODUCTION
 * Brief overview of SANDF CB Defense Policy
 * Definition of chemical/biological warfare
 * Brief historical overview
 * Ancient times
 * WW 1
 * WW 2
 * Cold war period
 * The Gulf crisis
 * Current threat (Post CWC period)

2. AGENT CHARACTERISTICS
 * Elementary introduction to organic chemistry
 * What is organic chemistry?
 * Unique properties of carbon
 * Bonding in organic compounds
 * Classes of organic compounds - homologous series and isomerism
 * Alkyl and functional groups
 * Naming organic compounds - systematic and trivial nomenclature
 * Alkyl phosphonic acids and derivatives - structural relationships between G and V agents
 * Definition of chemical warfare agents
 * Different classifications
 * Type of effect (nerve, blister or vesicant, choking, blood, tear, psychochemical or mentally incapacitating, vomiting or sternutating)
 * Physical state (true gas or vapor, liquid aerosol or mist, solid aerosol or smoke, liquid contamination)
 * Duration of effect (persistent, non-persistent)
 * Degree of effect (harassing, incapacitating, lethal)
 * Specific examples according to different classifications
 * Symptoms of exposure

* Definition, overview and classification of biological warfare agents
* Overview of riot control agents

3. DISARMAMENT AGREEMENTS
   * CWC
   * BTWC
   * Implications of disarmament agreements for SANDF

4. THREAT DEFINITION
   * International
   * Influence of disarmament agreements
   * States parties/Non-States parties
   * National
   * Intelligence (principles/resources)
   * Conventional
   * Terrorist

5. PROTECTION
   * Basic principles of protection against CB weapons
   * Individual protection
   * Definition
   * Respiratory/body protection
   * Immediate reaction drills (to be referred to only)
   * Equipment/maintenance
   * Carbon/filters
   * MOPP levels/threat levels
   * Operational constraints/factors influencing operational functioning
   * Command/control
   * Specialist equipment
   * Collective protection
   * Definition
   * Types (buildings/facilities/vehicles/ships/temporary (with consideration of operational constraints)

6. DETECTION
   * Definition/motivation
   * Tactical considerations
   * Alarm/control/mobile detection
   * Detection methods
   * Overview of different detector types
   * Maintenance requirements
   * Chemical reconnaissance
   * Chemical sentries

7. DECONTAMINATION
   * Definition and types of decontamination
   * Decontamination approaches and methods
   * Personnel
   * Equipment
   * Vehicles
   * Terrain
   * Casualties
   * Decontaminants
   * Principles governing organization, planning, appreciation, lay-out and operation of a decontamination center
   * Decontamination systems and equipment

* Influence on operations

8. INVESTIGATION OF ALLEGED USE
    * Need
    * Planning
    * Interviewing
    * Sample types and collection procedures
    * Sample handling/care/chain of custody
    * Reporting

9. FACTORS GOVERNING THE USE OF CHEMICAL WEAPONS
    * Methods of dispersal including application of bursting munitions
    * Agent characteristics
    * Delivery systems
    * Weather
    * Terrain
    * State of training/protection of opponent forces
    * Logistic burden to user
    * Duration of effect
    * Tactical aspects of the use of CW

10. CB CELL
    * Composition and planning
    * Communications
    * Downwind hazard and computer model
    * Cloud characteristics and integration of the above in appreciation

11. DEFENSE AGAINST CHEMICAL WARFARE
    * Threat levels (lethal, incapacitating, harassing)
    * Mapwork
    * Zones
    * Models
    * Computerized models/simulation
    * Tactical application of chemical weapons
    * Influence on own forces
    * Influence of weather/terrain
    * Communications
    * Chemical alarms
    * Planning
    * Appreciation of how, where and when chemical munitions will be used, and how defending force should employ countermeasures
    * Decontamination appreciation

12. CHEMICAL DISASTER MANAGEMENT (HAZMAT)
    * Role/responsibility of SANDF in chemical disasters
    * Advice
    * Hazard prediction
    * Detection
    * Decontamination

CONCLUSION

The first course was presented on a trial basis during October 1999, and was attended by a small number of candidates, who expressed general satisfaction with the overall content and level of presentation of the different sections. In particular, the introductory section on basic principles and concepts of organic chemistry was found to

be interesting and informative, and was deemed essential for a proper understanding of the overall course content. Certain sections of the curriculum could still be extended within the time span that is available for the course as a whole.

Lectures were shared between scientists from Protechnik Laboratories and officers of the South African Military Health Service, whereby both the technical aspects and the operational application of the course content were covered adequately in a joint venture.

The curriculum is subject to annual revision. In particular, biological defense had previously been neglected in courses of this nature, owing to the lack of a clearly defined biological threat from a South African perspective. The growing worldwide concern over possible biological weapons proliferation, also from third world countries who may gain access to the necessary technological and other requirements thereof, will necessitate increasing emphasis on biological defense and related issues during future revisions of the curriculum.

The international tendency to do away with traditional distinctive boundaries between chemical, toxin and biological warfare agents has resulted in these boundaries becoming increasingly diffuse, so that such weapons are currently being considered as part of a continuous spectrum. This calls for an integrated approach to the development of defensive measures against them, including training programs, as opposed to the treatment of such weapons as separate and unrelated entities. A start in this direction has been made in the compilation of this training curriculum, which will be further extended during future revisions of the curriculum content.

This course was designed according to specific needs and requirements of the South African National Defense Force. However, cooperation with other interested countries in respect of training may be considered in future. A diverse profile may therefore be encountered from candidates coming from different cultural and educational backgrounds, also from within the current SANDF with its ethnic diversity.

KEYWORDS
Defense, chemical, curriculum, training, advisers

59. AGROCHEMICALS: A TOXIC TERROR IN SOUTH AFRICA

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In agriculture, around one billion workers are employed word-widely, which is about 50% of a total world workforces. The third world suffer 50% of global poisoning and 75% of worlds agrochemical related fatalities, despite using only 20% of worlds agrochemicals. In South Africa, agrochemicals are widely used, and the agrochemical poisoning, negative environmental impacts are significantly under reported The ever increasing, urban, industrial, mining and agricultural development throughout the world is leading to levels of basic needs of food, housing, and sanitation. All in turn leads to a high level of potential exposure of flora, fauna and human to psycho-socio--chemical which seriously threaten the natural resources and human upon which the human and fauna depend for their survival and subsistence. Farm family and farm workers and the rural residents may be exposed to agrochemicals both directly (application of agrochemical) and indirectly (living in the agricultural areas). The users as well as the public have some minimal knowledge of the toxicity, protection and perception of risk of agrochemical. Present flooding and change of climate and outbreak of Malaria in Southern Africa, the use of the most dangerous chemical is initiated again. Therefore, the chemical toxic terror will continue devastating our resources. New constitution of South Africa puts the obligation on government to ensure the right of all South African citizens, to a clean and healthy environment. This presentation reviews the agrochemical toxic situation in South Africa, particularly, with respect to financial, educational, and law enforcement limitation.

Keywords: Agrochemical, toxic chemicals

60. THE SPANISH ARMED FORCES AUTOINJECTOR FOR NERVE AGENT ANTIDOTE

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Detachment Number 2 of the Central Pharmacy Park, in Córdoba, is in charge of manufacturing parenteral solutions for the Pharmaceutical Services of the Spanish Armed Forces. One of its main projects is R+D and manufacturing of prefilled syringes and autoinjectors for NBC defence. The S.A.F. Autoinjector contains 2 mg of atropine sulphate and 220 mg of obidoxime chlorhidrate in 1ml. This paper aims at explaining its functioning, manufacturing process and technical characteristics. New directions include: 1. Replacing 1 ml syringes with 2 ml syringes. 2. Introducing HI-6 autoinjectors (R+D of two chambers autoinjectors). 3. R+D of atropine+oxime+anticonvulsant autoinjectors. 4. Possibility of formulating autoinjectors with other drugs.

Key words: atropine, autoinjector, nerve agent, obidoxime

61. DECONTAMINATION OF CASUALTIES AFTER EXPOSURE TO HARMFUL LIQUID CHEMICALS

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INTRODUCTION

There are considerable risks of chemical accidents in industry and during transport. In addition there has been a growing concern about the possibility of terrorist use of biological or chemical agents. Furthermore by participating in humanitarian, peacekeeping or peace enforcing missions to different countries a number of civilians and military personnel will run the risk of exposure to known or unknown chemicals.

In 1992 the National Board of Health and Welfare (SoS) in Sweden established priority guidelines for measures to protect the medical and health care services against nuclear and chemical warfare agents. The Board also took the initiative to promote thorough discussions on chemical protection in medical care between civilian and military authorities concerned with the matter. These discussions resulted in a report (1) in which measures were suggested for medical personnel at emergency departments, medical teams and ambulance crews to receive personal protective equipment which will afford them at least limited ability to deal with chemically contaminated patients at the accident site, during transport and on admission to a hospital.

In this contribution I will review some projects encouraged and financially supported by the National Board of Health and Welfare and Swedish Agency for Civil Emergency Planning (ÖCB).

FUNCTIONAL ASSESSMENT OF THE DECONTAMINATION UNIT AT THE STOCKHOLM SÖDER HOSPITAL FOLLOWING EXPOSURE TO SIMULATED LIQUID PHASE CONTAMINANTS.

The objective of this study was to evaluate how efficiently volunteers exposed to simulated liquid contaminants could be decontaminated (2). Twenty-four volunteers participated in the experiment. Eight volunteers presented ambulant cases and sixteen on stretchers. They were all fully dressed and all were wearing a protective mask. During the experiment all staff was wearing personal protective equipment including respiratory protection (M90), dress 701 and rubber gloves and boots. The clothes, skin and hair of the volunteers were contaminated with the simulated liquid phase contaminants, ethyl lactate and methyl salicylate. Ethyl lactate is completely water soluble with properties but not toxicity, similar to sarin. Methyl salicylate is poorly soluble in water and not very volatile. It is a nasty-smelling, familiar component of liniment that could cause some local irritation. Sulphur hexafluoride gas was used to confirm the ventilation efficacy. Decontamination followed the guidelines using a two-stage procedure. In the first chamber, all volunteers received a 3-minute shower with water at 30°C, and their clothes, but not the respiratory masks, were removed. In the second chamber they were twice washed thoroughly with soap and water. After decontamination, the volunteers entered a third chamber for first aid measures. The capacity was 16 volunteers per hour with two thirds on stretchers. The air concentration of sulphur hexafluoride was reduced by 1:10,000 between the first and the third chamber. The levels of ethyl lactate and methyl salicylate measured in the third chamber were low (around 5_g/m^3). However, after self-decontamination of the staff the concentration of ethyl lactate increased significantly in the third chamber. One explanation to this increased level could be residual ethyl lactate in their underwear (a kind of cotton pajama). This observation revealed a deficiency in the guidelines for self-decontamination.

TO DECONTAMINATE OR NOT TO DECONTAMINATE...?

The scope of this study (3) was to examine whether residual toxic gases from clothes of casualties accidentally exposed to industrial chemicals could be transferred to an ambulance or an emergency ward to such an extent, that ambulance crew and medical personnel could risk becoming chemical casualties themselves. It could be expected that the risk is high for contamination by residual amounts of toxic gases in clothes, if the chemical accident happens in an industrial facility with a limited space. The study should also give information on how these toxic gases were transferred. A fully dressed dummy, warmed to a temperature similar to a living human being, was exposed to ammonia, sulphur dioxide or chlorine at

various concentrations (1000, 10,000 100,000 ppm) in a small chamber. After exposure for 15 minutes the dummy was carried to an "emergency ward", where the levels of evaporating gas were measured. The measurements were made 30 cm above the chest of the dummy, since this distance ought to be a fairly good estimation of the risk zone of medical staff (nurses) for exposure to toxic gases by inhalation. The method was verified for human conditions by exposing one volunteer for the lowest concentration of ammonia (1000 ppm).

The results of this study show that chemical casualties exposed to high concentration of toxic industrial gases could bring concentrations, high enough to harm the medical staff. In extreme cases the medical staff could be so seriously affected that they have to be subject to emergency treatment.

The medical personnel taking care of chemical casualties are recommended to wear respiratory protection before the patients are undressed. In cases when it is obvious that casualties have been exposed to low concentrations of industrial gases there is no need for undressing of the exposed or protective equipment.

PERMANENT FACILITIES AT HOSPITALS FOR DECONTAMINATION OF CHEMICAL CASUALTIES – VALIDATION OF FUNCTION AND ROUTINES

In Sweden, the National Board on Health and Welfare supports the authority responsible for the hospitals, county councils, financially and by expertise to implement a program to establish permanent decontamination units at emergency hospitals. Today about 50% of the emergency hospitals have obtained such facilities. It is estimated that in a couple of years all emergency hospitals will have facilities for decontamination.

The decontamination units at the emergency hospitals differ from the unit at DEMC, Stockholm Söder hospital (SÖS). In this three-chamber unit, decontamination is performed stepwise. It is constructed for high capacity and security and for decontamination of casualties exposed to highly toxic agents in peacetime and during war conditions.

The decontamination facilities at the majority of emergency hospitals are designed not only for accidents with chemicals but also for substances giving ionizing radiation under peacetime conditions. They should also be safe enough to allow decontamination of casualties contaminated with small amounts of highly toxic chemicals, i.e. chemical warfare agents (CWA) without causing serious hazards for medical personnel, patients or various other functions at the hospital. The facilities are built for security and low capacity and are suitable for daily cleaning purposes.

The decontamination of chemical casualties near the accident scene is of major importance, while the decontamination unit at the hospital chiefly should fulfil the need to decontaminate contaminated chemical casualties appearing spontaneously at the hospital. The capacity and routines of the decontamination units at the emergency hospital are dimensioned for decontamination of a limited number of casualties simultaneously. However, the hospital will have to decontaminate and take care of a large number of casualties. This situation will of course put a heavy burden on the hospital.

There is also a need for education and training of the medical personnel at the emergency hospitals, who are tasked with the decontamination. The education and training available today are usually either based on decontamination in the field or decontamination in the advanced three-chamber unit of Stockholm Söder hospital.

Roughly described, the facilities at the emergency hospitals usually consist of a decontamination room, a lock and an ambulance hall. However, there are differences between the various hospitals. It therefore appeared important to validate the function and routines at least in some of the decontamination facilities to try to make them optimal for the facility. It is also important to give the medical personnel instructions, education and training adapted to the facility in which they will perform the decontamination of chemical casualties.

The National Board of Health and Welfare therefore took the initiative to a study of the function and the routines of the decontamination facilities. Of the units selected, two were located in emergency hospitals and one in an outpatient care center. One facility was situated in the south of Sweden; the other two were in the north. The selection was made so that the selected facilities were representative of the facilities built in Sweden. The studies were designed to answer some important questions, for instance: How dangerous is it to get significant amounts of highly toxic agents into the facility? How high is the risk for unintentional distribution of chemicals from the decontamination facility to other rooms of the hospital? When, after completed decontamination, can the decontamination facility be considered clean?

The selected facilities were studied during both summer and winter conditions. The design of the study was similar to the study of the decontamination facility at the Stockholm Söder hospital but a great number of new parameters were measured. Before performing experiments with human volunteers, the function of the facilities was studied. Ventilation, water supply, drainage, and control systems were among the parameters studied. Technical shortcomings were taken care of when possible.

Before the experiment the medical personnel tasked with the decontamination obtained education and training on the protective equipment. In the experiment the volunteers, usually in groups of 3 or 4. were exposed to the simulated liquid phase contaminants ethyl lactate and methyl salicylate. They were then taken into the decontamination room and decontaminated with soap and water and taken through the lock and the ambulance hall into the hospital (or ward). The levels of the contaminants in the air of different rooms were continuously monitored or sampled at intervals for analyses. A vast number of other parameters were also measured. The results of this study are now being compiled in a final report. It can be safely concluded that the facilities studied, with some changes in instruction and routines, are functioning well both during summer and wintertime. The medical personnel have obtained education and valuable training under realistic conditions. Furthermore, valuable information has been obtained for preparing guidelines for the facilities built and to consider seriously, when building new facilities. The result should also be of value in the education and training of medical personnel.

GENERAL CONCLUSIONS

Some of the efforts made in Sweden to increase the protection of the hospital against chemical accidents and chemical warfare by improving the ability of the Medical and Health Care to handle Chemical casualties and to increase its protection against chemical contamination have been reviewed

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62. EVALUATION OF METHODS FOR INTERLABORATORY COMPARISON TESTS ON MEASUREMENTS OF CHOLINESTERASE ACTIVITY

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Measurements of total cholinesterase activity are widely used for assessing the severity of organophosphorus intoxication. Currently different methods are in use resulting in values that directly can not be compared with each other. In the future it would be of great help if different laboratories would use one and the same method and express the results in the same units. In the present study, we have determined acetyl- and butyrylcholinesterase activities (AChE and BChE, respectively) in samples drawn from capillary (finger) and in venous blood. In order to standardize enzyme activity they were related to the hemoglobin content of the blood samples determined without or with conversion of hemoglobin to hemoglobin cyanide by the method of Zijlstra. In addition we compared the effect of two detergents, Triton X-100 and Pluronic F 68, on AChE and BChE activities as determined in samples drawn from finger blood.

Keywords: cholinesterase, organo-phosphorus intoxication, screening,, erythrocytes chemical warfare agents, biomarker, inhibitor

63. THE SWISS WAY TO COUNTER CHEMICAL TERRORISM

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After the sarin attempt in the Tokyo underground, the Swiss population started to ask about the possibility and consequences of such an event in our country. A working group was nominated by the national board for NC protection and was given the task to assess the situation. The conclusion was that the low probability would not justify keeping the population under continuous alert.

Therefore it was recommended, e.g.:

- integrate the problems of chemical terrorism into the concepts of protection against chemical risks,
- check the equipment of the emergency responders,
- check availability and suitability of military chemical protective equipment for emergency responders (respirators, suits, CW detection instruments, antidotes),
- nominate laboratories and coordination bodies for criminal investigation and information/documentation.

The army material is being readied for supply to the emergency organizations, as soon as the training in application has taken place. This is the most cost-effective way of upgrading the equipment of civilian emergency organizations.

Discussions with the cantonal authorities demonstrated a need for support by a national expert team from the AC-Center Spiez. It was organized in 1999 and equipped with the most modern protective outfit, detection devices, etc. Depending on transport means, it will arrive at the site within hours of the event. Part of the team will remain at the AC-Center to provide data and information and to prepare the analysis of samples, which are brought to Spiez as quickly as possible.

The poster gives information about the organization and the equipment of the expert team and the coordination of activities on site.

Keywords: CW detection, protection, chemical preparedness, civilian preparedness

64. TOWARDS A COMMON METHOD FOR MEASURING CHOLINESTERASE ACTIVITY

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In occupational health, the estimations of the activity of acetyl- as well as butyrylcholinesterase give a good indication for intoxication with insecticides, especially when normalized to the hemoglobin content of the sample. The same activity measurements are also of importance in the treatment of heavily intoxicated patients, since this allows the follow up of the efficacy of the treatment. Several groups agreed, during a special CBMTS meeting in 1997, in important methodological steps towards developing a common accepted method. This method had to be worked out and a kit was shipped to different laboratories. The outcome of the results showed a relative high variation in butyrylcholinesterase and in hemoglobin content.

The variation in hemoglobin measurement was clearly due to the loss of hydrocyanic acid in the ready reagent contained and shipped with the kit.

The variation in the butyrylcholinesterase turned out to be an artifact resulting from the variable inhibition of this enzyme by the detergent Triton X-100. In our original assay at the AC Lab., we were lysing the blood with quartz distilled water, but not adding a detergent. Triton X-100 was being used by several laboratories in their cholinesterase measurements. We did not at first think to check the detergent effect on both enzymes individually and compare it to the lysis with water. We have now tested some detergents for their effect on both enzymes, as well as on the estimation of the hemoglobin. The detergent concentration used was in each case above the critical micelle concentration. These results and the resulting modified method will be presented.

Keywords: cholinesterase, organophosphorus intoxication, screening,, erythrocytes chemical warfare agents, biomarker, inhibitor

65. MONITORING OF POTENTIAL HEALTH EFFECTS OF NERVE AGENT DESTRUCTION IN SHCHUCH'YE, KURGAN OBLAST (SOUTHERN URALS), RUSSIAN FEDERATION

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INTRODUCTION

Russia is the world's largest possessor of chemical weapons. Over 40,000 metric tons of chemical agents are stored today at seven sites. In accordance with the Federal Programme on Chemical Weapons Destruction, decreed on March 21, 1996, and with the Chemical Weapons Convention ratified by the Russian Federation on November 4, 1997, the Russian chemical weapons will be destroyed on site in special destruction facilities.

In Shchuch'ye (Kurgan Region, Southern Urals), the first destruction facility for nerve agents will be built with the support of the American CTR Programme. Approximately 5'450 t of phosphor-organic agents (Sarin, Soman, and VX) in artillery shells and missile warheads will be destroyed with the so-called 'two-stage destruction technology.' In a first step, the toxicity is reduced by mixing organic solvents with the warfare agents; in a second step the process is made irreversible through bituminisation of the salt mass. At the moment final tests are investigating the long-term properties and (non-)toxicity of the bitumen mass. Construction start is scheduled for 2001 and operation start-up for 2008.

Main concerns of the communities living around the stockpile centre on health, state of the environment and emergency preparedness. A ubiquitous believe in all stockpile areas is that the bad state of public health is related to the presence of the chemical weapons. In order to address these concerns and as a baseline to monitor future trends in health, a report on the health status of the population living near the Shchuch'ye chemical weapon storage facility has been prepared in 1997 by a medical group under the leadership of Dr. Tatyana Grozdova (Saratov, Russia). In the same year, the Institute for Social and Preventive Medicine, University of Basel, Switzerland, was requested by Green Cross Switzerland a) to provide advice to the Grozdova group on the assessment and preparation of existing epidemiological data, b) to define gaps of knowledge, c) to support the application of recommendations in two previous reviews of the report (Abelin 1997, Henderson and Joe 1997); and d) to support and advise the group on the development of a long-term health monitoring concept.

METHODS

This advisory report is based on the following materials and methods:

(1) Personal observations and discussions with persons involved in the project during a visit of Dr. R. Kaiser to Moscow and Shchuch'ye from 23 - 31 January 1998, including public hearings in Shumikha and Chumlyak and visits to hospitals and ambulatories in Shchuch'ye, Chumlyak, Planovye, and Peshanskoye;

(2) Personal communication with experts in relevant fields; and,

(3) Review of available international literature (see References).

RESULTS AND DISCUSSION

Shchuch'ye is a town of 10'600 people, located about 1600 km east of Moscow. The administrative area has 2'858 km^2. Population density is low (10/ km^2). It is a predominantly agricultural area with some industry in the town of Shchuch'ye. The socio-economic situation has deteriorated in recent years with reduction of incomes and increase of unemployment: today only three enterprises still provide employment.

The largest contribution to air pollution in the Shchuch'ye area stems from 19 coal and fuel operated boiler-houses. The most hazardous combustion substances are lead and its inorganic compounds, vanadium pentoxide, nitrogen oxides, and sulphur. Although limited in number, motor vehicles are also a major source of lead pollution. There is no air-monitoring programme.

The area's water resources are deficient and of poor quality and there are polluted surface and underground water sources. Underground waters are the main source of water supply for the enterprises and the population of the Shchuch'ye area. About 40% of water is delivered through pipes, the remaining 60% stems from open wells. Most of the settlements in the area do not have a centralised water supply and sewage system nor are there water treatment facilities for industrial and household effluents.

The Shchuch'ye area has 90 waste disposal sites (dumps, manure-pits, ash-slag heaps, animal burial grounds. etc.). These sites are operated without any regulatory oversight or environmental monitoring.

Another source of environmental contamination is the Mayak plutonium breeding facility. Its operation has resulted in major radioactive releases into the environment and significant overexposures for thousands of workers and the nearby population. Though the Shchuch'ye area is situated outside of the main contamination plume, medical personnel are concerned about a relationship between a number of lung cancer cases and potential radiation exposure.

The Grozdova report (Grozdova et al 1997) showed little difference in the prevalence of diseases among the residents of Shchuch'ye, Chumlyak, Peschanskoe, and the Kurgan Region. The reviewers of the report, however, challenged the validity of the data for the following reasons:

a) lack of financial resources: The operation of hospitals suffers from underfunding, salaries are paid only on a delayed basis, several doctors left the Shchuch'ye area, and patients have to buy and bring medical materials themselves.

b) limited diagnostic equipment: Diagnostic equipment is very simple and limited to a rarely used ultrasonographic instrument and a fiberoptic gastroscope.

c) underuse of health care facilities: Many people do not use the health care facilities and do not travel to the Shchuch'ye central hospital, even if referred, because of cost. It has been observed that patients come at stages of advanced disease when treatment is hardly successful.

d) inherent limitations in the health reporting system: Most people are registered at local nurse stations for medical care. Thirty-seven of these nurse stations are spread over the low populated area. In case of more complicated medical problems patients are referred to the peripheral hospitals or the central hospital in Shchuch'ye. Severe cases are generally treated in Shchuch'ye. A system of mandatory annual health reporting had been established in socialist times. The number of case episodes (for acute diseases, such as temporary respiratory diseases) and patients seen (for chronic diseases) are collected through referral and then compiled in Shchuch'ye for the annual health report. All patient information is written, reported and summarised by hand. The main limitations of health reporting are underestimation of the overall prevalence of diseases and misclassification of diseases due to low diagnostic standards.

One major concern of the population in the Shchuch'ye area is a possible deterioration of the general state of public health after the start-up of the chemical weapon destruction facility. However, monitoring the population for diseases related to releases of nerve agents from the Shchuch'ye facility is not considered to be a useful approach because nothing in the current literature on human and animal studies indicates any long-term health effects associated with low exposures to nerve agents.
Even with an exposure large enough to affect disease rates, detection of changes for rare outcomes would be difficult due to the small population studied and the low level of diagnostics. This can be exemplified by a hypothetical case assuming a radiation exposure in the northern part of the Shchuch'ye area: if the incidence for cancers of the blood and lymphoid system in the Shchuch'ye area doubles from 1996 to 2006, the number of cases would be 10 instead of 5 cases. As a result, there might be concerns about a link between ionising radiation and cancer of the blood and lymphoid system. The magnitude of increase, however, would not considerably differ from the range of 0 to 9 cases that were found within the period from 1988 to 1996. Thus it will not be possible to statistically separate a true increment from expected data variability.

Since exposure levels are expected to be low and effects are unpredictable, the appropriate design for a health investigation is that of occupational monitoring at the chemical weapons destruction facility. First, workers are expected to have the highest - if any - exposure; second, it is feasible to closely monitor and follow-up workers over a long period. A control group should be selected, if possible, from another exposure category within the destruction facility, or from another factory with a profile similar to the destruction facility.

However, an occupational monitoring programme should be an integral part of a general public health concept. Otherwise, the interpretation of occupational surveillance data could be compromised by adverse effects due to poor health conditions or lack of public health education (e.g. drinking, smoking). Health reporting is a valuable instrument to identify long-term trends in the overall health status of the population and to detect acute disease outbreaks. However, certain standards need to be adopted to improve the quality:

(1) **Diagnostic standards:**
If health care utilisation data are used for reporting it is essential to achieve the same standard of diagnostic equipment, diagnostic criteria and disease coding along the entire way of data collection. Repeated training may help to standardise this process. Although the 10th version of the international classification of diseases (ICD10) is currently being introduced in Russia, the disease classes of the 9th version may be kept until there is some experience with the new structure of ICD10.

(2) **Demographic population data, age standardisation:** Health reports provide us with health indicators such as mortality and morbidity in relation to the size of the population or population groups of different age and sex. Therefore, it is essential for health reporting to have access to annual data on population age groups, further differentiated by sex, and to use age standardisation techniques (direct and indirect) for comparison.

(3) **Presentation as population related rates:** Health reports should show demographic information (total population and age specific groups), birth, mortality and morbidity rates as total numbers and for different age groups (differentiated by sex) and by disease categories. Data sources have to be clearly stated. Missing values should generally be marked as such (e.g. n.a. = not available) and clearly differentiated from zero values.

Last but not least, public health education increases awareness in the population on the relevance of major health threats such as poor living conditions, poor health care and behaviours such as smoking and drinking. It should also help to efficiently prioritise the allocation of limited resources for improving general public health.

SUMMARY

The low standard of diagnostic equipment, the lack of demographic population baseline data and unexplained variations in morbidity rates indicating incomplete health services data were all identified as major limitations for reliable health reporting. Reliable public health data, however, are a prerequisite to identifying changes in morbidity or mortality patterns in populations.

Potential releases of nerve agents or destruction products into the environment would be low and chronic. Therefore, risk assessment among the total population may not be useful, because the population size is small and, according to current knowledge, it is unlikely that an exposure to nerve agents will play an important role in morbidity processes. The investigation of effects of low levels of nerve agents and their potential degradation products should focus on the occupational setting where exposure would be - if of any significance - the highest. The occupational epidemiological approach includes a) exposure assessment at the destruction facility and the bitumen disposal site, b) health monitoring of workers, and c) the assessment of cofactors of adverse health outcomes.

In general, the sustainable improvement of public health should be a major priority as it is likely that poor living conditions and standards of medical care have a far larger impact on the health status of the population and on limiting life expectancy. Occupational surveillance is considered to be an aspect within this broader scope. Improvement in the quality of health reporting is a prerequisite for receiving reliable data for determining changes in baseline conditions. Continuous and comprehensive public health education is recommended as a vessel to address potential hazards of the destruction facility, to teach actions to take in case of an exposure, and to enhance the general understanding on public health issues including disease prevention and health promotion.

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KEYWORDS
Shchuch'ye chemical weapons destruction facility, epidemiology, nerve agent destruction, occupational monitoring. health reporting.

66. IMMUNODETECTION OF BIOLOGICAL AGENTS BY REPERTOIRE CLONING

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INTRODUCTION

There is still a need for the development of rapid and sensitive detection methods for biological warfare agents and toxins to encounter appropriately the possible threat. A quick and specific detection system will be also useful in view of a future verification regime of the Biological Weapon Convention. The aim of the project is to evaluate and establish two semi-synthetic **antibody libraries** (Griffin.1 and ETH-2) by **phage display technology** that provides a means of capturing a fast diversity of monoclonal antibodies (Ab) by **repertoire cloning**.

In parallel, the conditions for use of selected Ab in diagnostic tests have to be improved by the development of adequate immobilization systems for the antigens (Ag). In addition to *Francisella tularensis*, which was chosen as a model Ag because of its BW-relevance, other antigens are also investigated. By repertoire cloning it should be possible to generate Ab against any biological agent without using laboratory animals.

Antibody library (Figure 1)

The immune system produces Ab which mark foreign molecules (Ag) for destruction. Each Ab is specific for each Ag (key-lock principle) and consists of two heavy (orange) and two light (yellow) chains. Although the number of different genes coding for the chains of an Ab is restricted, several million different variants are produced. For each variant, only a few genes for heavy and light chains are selected and newly recombined which leads to this variety. Molecular biology and genetic engineering techniques offer a new possibility for Ab production. They allow to create gene arrangements artificially and to store them in a library with the resulting repertoire being bigger than in nature. The genetic information for each Ab variant is inserted in a vector that can replicate. Bacteriophages are suitable vehicles for this purpose.

Phage display technology

A bacteriophage (or for short: phage) is a virus which infects bacteria and only replicates inside of the host cell. It can easily take up foreign DNA molecules such as genes for Ab which are subsequently presented („displayed") on the surface of the head. Each phage displays only one Ab variant. The entire Ab variety of an artificially constructed Ab library is produced and presented by an equivalent phage population.

MATERIAL AND METHODS

Construction of a phage display library by repertoire cloning (Figure 2)

1) Total RNA is isolated from peripheral blood lymphocytes

2) Ab-specific mRNA coding for heavy and light chains is selected and converted by reverse transcriptase polymerase (RT-PCR) to cDNA

3) The resulting cDNA corresponding to heavy and light chain parts is amplified by PCR

4) The genes for cloning into plasmids (ring-shaped DNA molecules) randomly recombine heavy and light chain parts of an Ab

5) The approximately 10^9 different DNA plasmids are subsequently introduced into phages

6) Each phage gets a specific variant, which is unique for a specific Ab. The phages expose their Ab on the surface of their heads

7) Selection of specific Ab (Ig= Immunoglobulin). The phage Ab library is screened for specific antigen-binding properties by subjecting the phages to repetitive rounds of selection (panning) which includes binding, washing and eluting steps from a source of solid-bound Ag (phage ELISA)

8) Propagation of the phages by infection of bacteria

9) Large-scale production of the specific Ab (scFv= single chain variable region) in bacteria and purification.

Screening

After 5-6 rounds of panning, ideally a population of phages was selected which was specific for the Ag under investigation. However, there was always a small fraction of selected phages that reacted non-specifically. Specific phage clones were identified by the phage ELISA and confirmed by DNA sequence analysis of the inserted Ab genes.

Interestingly, it was not always possible to select Ab against each Ag from both libraries. They were mixed together before the screening and the „best" Ab was selected in the panning. Thus it is favorable to investigate several libraries in the same screening.

Unfortunately no specific Ab could be found against CD46 and NS1. Since each Ag was only investigated once. altered screening conditions would probably be successful. However, the most possible explanation is denaturation of the Ag molecules during the adsorption to plastic (coating of microtiter plates). In this case the selected Ab recognizes only the denatured form of the Ag but not the native structure.

The denaturation of the antigen during the immobilization step can also happen partially, leaving some epitopes of the antigen in an intact form. This was probably the case for FOM and GST17, leading to specific Ab (positive capture ELISA). In further experiments it must be tried to derivatisate (biotinylate) the antigens in a way that screening can be done without the problem of denaturation.

RESULTS

Antigens used for screening

The two semi-synthetic Ab libraries Griffin.1 and ETH-2 were combined and used for selection of Ab by phage ELISA (Enzyme-Linked-ImmunoSorbent-Assay) against the following Ag: See Tables 1, 2, and 3.

Capture ELISA

The capture ELISA (also called "Sandwich ELISA") was developed to further test the selected Ab after the phage ELISA. The main advantage is the prevention of denaturation of the Ag since it is not directly coated onto the solid as in the phage ELISA, but captured in a liquid phase. A positive capture ELISA means that the selected Ab is directed against native epitopes of the Ag; a negative result points to denaturation of the Ag during previous selection of the Ab.

Principle

Figure 3

RSA is immobilised on a microtiter plate. ABP binds with high affinity to RSA. The selected Ab is expressed in E. coli as fusion protein with ABP. For this purpose the genes for the specific Ab (FOM, GST17, Rabies) selected in the phage ELISA were cloned in a gen-cassette containing either the ABP or the PhoA gene. When the specific Ag is added to the microtiter plate, the Ab captures it. After washing steps (not matching Ag is removed). the second. phosphatase-marked Ab is added.

A successful binding leads to a conversion of the phosphatase substrate that can be measured.

CONCLUSION

Our results indicate that the semi-synthetic libraries Griffin.1 and ETH-2 can be used for the selection of Ab against any biological agent. However, the immobilization procedure of the Ag has still to be improved by appropriate derivatisation steps in order to preserve the native structure.

The advantage of the presented approach is obvious and lies in the huge repertoire of the libraries. which is bigger than in nature. Moreover, no laboratory animals are used which is a big problem in conventional Ab selection when the Ag is toxic (animal dies) or non-immunogenic (no Ab).

OUTLOOK

The selected Ab will be further analyzed (diversity, specificity, sequence) and the ELISA systems improved as outlined above. In a next step the libraries will be used for the selection of Ab against cyanobacterial and other toxins.

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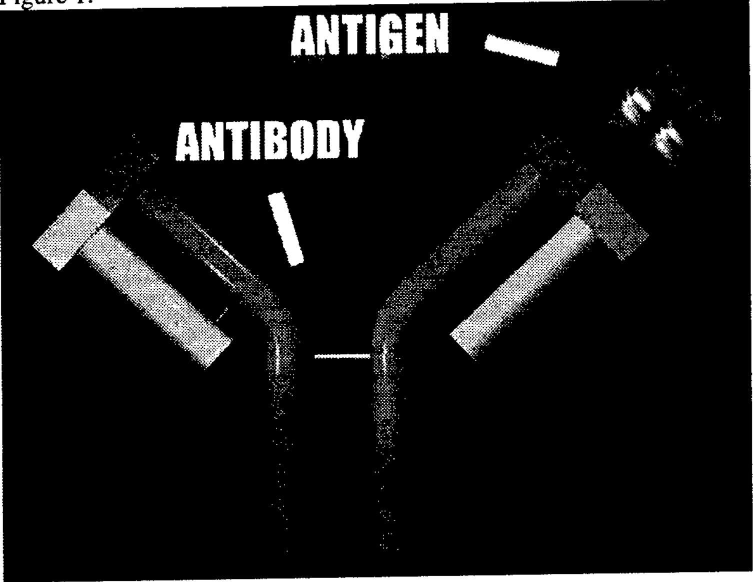
KEYWORDS

Antibody library, phage display technology, repertoire cloning

FIGURES AND TABLES

Figure 1.



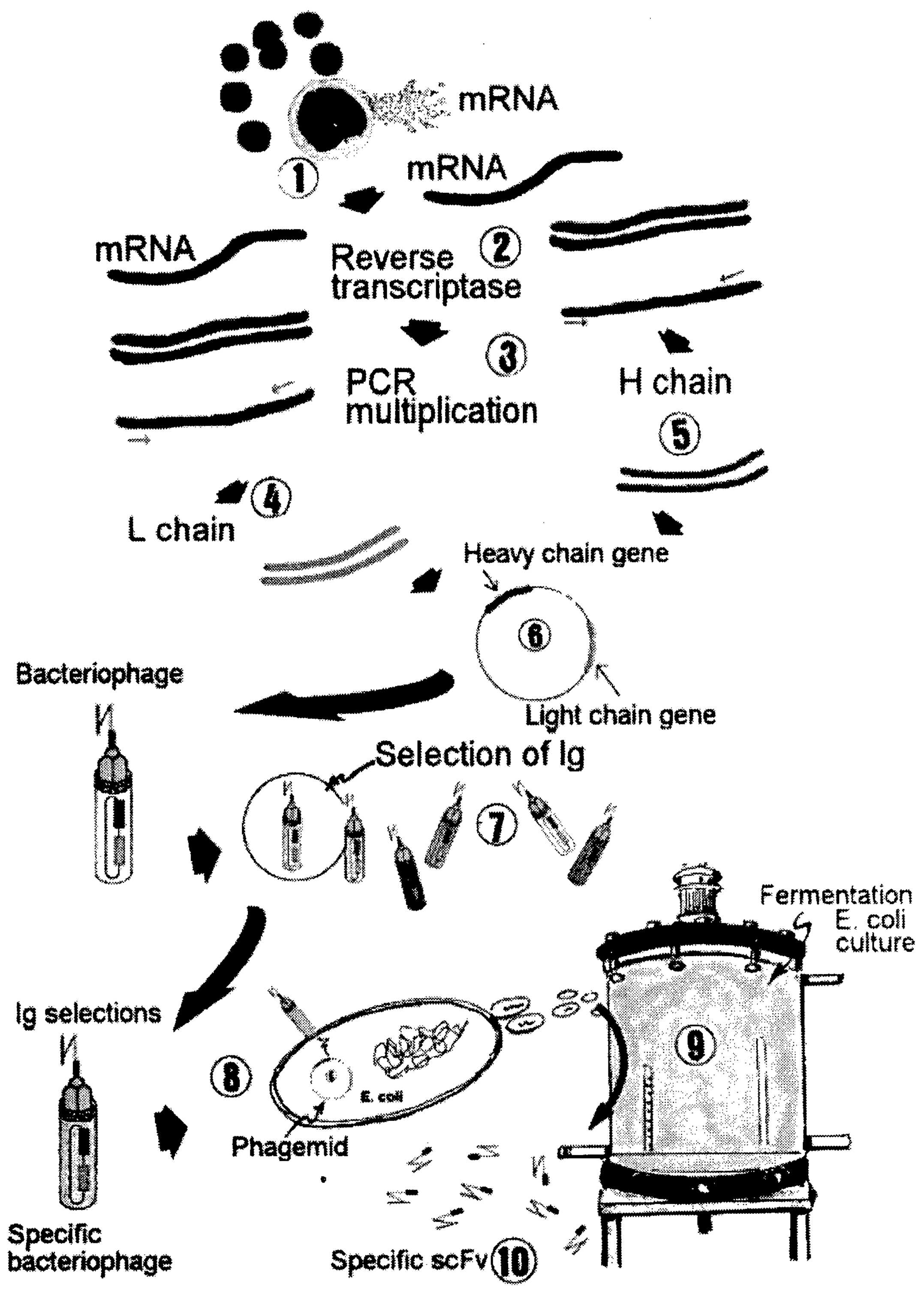


Figure 2.

Figure 3.

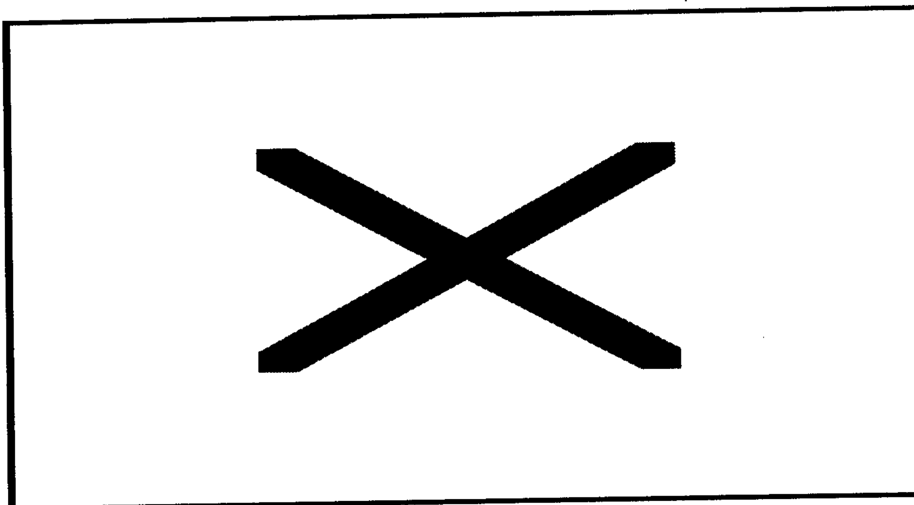


Table 1.

Antigen (Ag)	**Description**	**rounds of panning**	**Result (short form)**
FOM	Protein Fraction of the Outer Membrane of *Francisella tularensis*	6	specific Ab in the phage ELISA, positive also in the subsequent capture ELISA
GST 17	Recombinant Lipoprotein of *Francisella tularensis*	5	specific Ab in the phage ELISA, positive also in the subsequent capture ELISA
Rabies	Glycoprotein of rabies particle virus	5	specific Ab in the phage ELISA but negative in the capture ELISA (probably only specific against the denatured form of glycoprotein
Gfp	Green fluorescence protein	5	specific Ab in the phage ELISA, positive also in the subsequent capture ELISA
HSV-1	Herpes Simplex Virus 1 particle	4	positive but only weakly specific
CD46-eu	eukaryotic expressed measle virus receptor	4	unspecific positive
CD46-pro	prokkaryotic expressed measle virus receptor	4	unspecific positive
NS1	Non-Structural protein 1 of minute virus of mice	4	unspecific positive

Table 2.
Screen FOM

panning no.	output titer	polyclonal ELISA	monoclonal (phage) ELISA	Sequence
1	$2.3x10^6$			
2	$3x10^5$			
3	$9x10^7$	weakly positive	7 of 20 positive	
4	$3.9x10^7$	positive	3 of 20 positive	
5	$1.5x10^{10}$	positive	15 of 20 positive	4 clones sequenced: 1 unspecific ETH-2 2 identical ETH-2 1 Griffin.1
6	$2.7x10^6$	positive	13 of 20 positive	4 clones sequenced: All identical ETH-2 (as panning no. 5)

Table 3.
Screen GST17

panning no	output titer	polyclonal ELISA	monoclonal (phage ELISA)	Sequence
1	$8.5x10^5$			
2	$1.8x10x^5$			
3	$5.6x10^6$	positive		
4	$6.9x10^7$	positive	17 of 20 positive	5 clones sequenced: All identical Griffin.1
5	$1.5x10^8$	positive	14 of 20 positive	5 clones sequenced: All identical Griffin.1

67. DEVELOPMENTS IN MOLECULAR BIOLOGY AND THEIR IMPACT ON BW

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INTRODUCTION

Some personal thoughts about new techniques in molecular biology and their impact on the actual BW-threat and threat response are presented. These thoughts are mainly based

- on a short overview of some of the most relevant methods (technologies) and devices in molecular biology
- on a few samples of applications for BW-related studies, and
- on possible countermeasures for reducing the BW-threat

TECHNOLOGY

rDNA technology with all the related methods stands on first position. With the discovery of the DNA composition and structure, the different enzymes (restriction enzyme, ligases) and the possibility to transform organisms with foreign DNA, a broad field in bioscience has been opened. This started in the mid 70s; it was at that point when also fear about possible misuse of rDNA technology lead to an increasing interest in new biological agents as weapons. So this is really the key technology when it comes to BW threat. Other methods or technologies are of course also very important. Just to name some of the most relevant: PCR technology with its origin in the 80s. This is a very powerful tool for the rapid detection of agents. One of the different variations of PCR is the real-time quantitative PCR, e.g. the TaqMan-System. DNA-sequencing is a little bit older, but nevertheless still very helpful and powerful for agent characterization purposes. Since the HGP has been launched, a lot of progress has been made towards an easier and faster sequencing. New methods and technologies are only recently introduced such as the microchip technology, a system that combines molecular biology and microelectronics, with interesting tools for rapid detection and screening of unknown biological samples. Another system that is probably not yet commercially available on the market, is the so-called Invader assay. Similar to PCR, hybridization probes are used, but instead of temperature cycling, the hybridization complex is cleaved by structure-specific enzymes emitting fluorescence signals that can be detected. With this method specific DNA and RNA sequences can be rapidly detected.

Devices that operate on the basis of some of the mentioned techniques are the ABI-sequence detection system, the light-cycler, both for quantitative PCR, then a miniaturized PCR system, the smart cycler for on-site, very fast analysis and detection of biological agents. Furthermore, there is the very promising gene-chip system.

The application of new molecular methods or techniques goes in two directions:

- analytical studies (e.g. epidemiological studies or detection of pathogens, medical diagnostics and so on).
- genetic engineering or in a more broader sense basic molecular biology for research studies

The first category does not seem to be very sensitive in terms of BW-threat. It is rather a convenient means to rapidly detect potential BW-agents. The second category however, comprises a lot of sensitive basic research projects. One of them is the sequencing project of the human genome, the so-called HGP. This project is an international effort to discover and map all of the 60‘000-80'000 human genes and make them accessible for further studies. In addition the whole genome shall be sequenced. Until now 10% of the genes are mapped and less than 5% of the genome is sequenced. Very recently it was published that the chromosome 21 has been fully sequenced. It is clear that in the course of this project a lot of benefits for instance for the better understanding of genetic diseases will be generated. On the other hand there is already very sensitive information available about ethnical differences on the genetic level. Of course, HGP is by far not the only sensitive project. Each similar project dealing for instance with special human pathogens that are recognized as putative BW-agents may be very interesting from a scientific point of view and probably also necessary to better understand the mechanisms of pathogenicity or virulence. But sometimes they are also very close to offensive BW research projects. In fact it would be impossible to distinguish between their defensive or offensive character.

This is exactly the point of concern. Although, there are a lot of very promising developments from the application of new molecular techniques in the field of medical diagnostics, rapid detection of pathogens or basic research, it is obvious that with the same techniques or technologies a new element of BW-threat has been generated.

REDEFINING THE BW THREAT

So the question is: "What can we do to respond to this threat and perhaps also to prevent scientists from misusing their know-how?"

One element of threat response is obviously the establishment of national threat response programs and the international collaboration. A different approach comes from the Biological Weapons Convention (BWC). Although

the BWC has unfortunately not foreseen a verification regime, the convention nonetheless covers a key element under Article X, where it says: „The States Parties to this Convention undertake to facilitate, and have the right to participate in, the fullest possible exchange of equipment, materials and scientific and technological information for the use of bacteriological (biological) agents and toxins for peaceful purposes. [...]". The same element is again part of the mandate of the Ad Hoc Group in Geneva, which negotiate now the strengthening of the BWC with an additional verification regime. Both Article X of the Convention and Article VII of the future Protocol inherit to notion of collaboration and through that the possibility of building transparency and trust. Since the experience with UNSCOM in Iraq it was realized, that the BW-verification without a long-term transparency-, confidence- and trust-building system is very difficult. Of course, an efficient verification must stand on the three pillars of declarations, visits (also a sort of transparency measure) and challenge inspections. But it must be noticed, that the big problem of BW is neither the agent itself (as it may be the case for CW) nor research and development activities or different projects as such. It is rather the intention behind BW-related activities, which is essential for the distinction between prohibited offensive and allowed defensive programs. Therefore verification in the narrow sense of declarations, visits and inspections is certainly not good enough. The additional controlled exchange of know-how and equipment is extremely important for the entire verification system.

CONCLUSIONS

It is obvious however, that even the strengthened BWC will not be able to cover the whole BW threat spectrum. For further threat response it will be very crucial to count on the responsibility of the scientific and research community as a whole and also as a community of different individuals. Each of its members has to be aware of the fact, that probably not everything that seems to be scientifically feasible is also really worth to do. On the other hand it would be not wise to prohibit certain research activities. Prohibition normally does not work. It is much better to specifically improve the scientific knowledge and in parallel exchange the results on an international level. This is exactly the intention of the CBMTS and there is hope that this platform is another step forward to strengthen the consciousness for scientific community responsibility.

68. CAPSAICIN IN PEPPERSPRAY - MODE OF ACTION

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As a result of aggressive marketing, pepper sprays are widely accepted for use in law enforcement by military or police forces, as well as for private persons. A number of studies have tried to show the advantages of pepper sprays compared to teargases like CS or CN. But there are quite a number of critical points and open questions, which should have been solved beforehand.

The extraction conditions and methods for the hot pepper fruits influence the composition of the fractional part of Capsaicin containing homologues and other substances that may have different modes of action. The solvents, propellants, the mode of distribution and so on, are responsible for storage stability, effectiveness, and the risk of injuries. The description of action is sudden, but there are no determinations available of exact latency time.

The exact mode of action is still under research, but since about two years it is known that there are different vanilloid-receptors that are influenced by capsaicin. These receptors are responsible for the opening of the cation channels in nerve cells, which in turn lead to a massive Ca++-influx that may result finally in cell death.

The critical presentation of the lack of knowledge leads us to the same conclusion as mentioned in the Himsworth-Report: Irritants should be studied to the same extent as pharmaceuticals to be registered. This means, that the intended application on eyes and by inhalation must be shown to be effective and not harmful; unwanted side effects, especially on the vegetative nervous system and the CNS must be ruled out.

Keywords: capsaicin, pepper spray, riot control, irritants

69. CONSEQUENCES OF TERRORISM, SABOTAGE AND NATURAL DISASTERS ASSOCIATED WITH THE CHEMICALS USED IN PHARMACEUTICAL AND CHEMICAL INDUSTRIES

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INTRODUCTION

As large quantities of various dangerous chemicals are produced, stored, and transported during the manufacturing process, extremely dangerous conditions could exist within the complexes of chemical, petrochemical, pharmaceutical and other related industries. These dangerous conditions could lead to catastrophes when triggered or aggravated by acts of

- terrorism
- sabotage
- combat
- earthquake
- large scale incident or accidents (1).

Potential targets could be

- chemical industries manufacturing chlorine, peroxides, oxygen, organic chemical intermediate liquids and gases, plastics and pesticides.
- Food processing and storage facilities with the large ammonia tanks
- chemical transportation assets (i.e. Rail tank cars, tank trucks, pipelines)
- gasoline and jet fuel storage facilities at distribution centers, airports and barge terminals
- gold mines where cyanide and mercury compounds are used
- pesticide manufacturing and supply distributors, and educational medical and research laboratories (2).

The potential risk from releasing, uncontrollable spreading of high concentration of toxic chemical substances in these industries can lead to

- powerful explosions
- fires
- poisoning of humans and animals
- contamination of environment
- destruction of buildings and equipment.

Fire may produce irritating, corrosive and/or toxic gases. These vapors may form explosive mixtures with the air, and also they may travel to source of ignition and flash back. As most vapors are heavier than air, they may travel along the ground and collect in low or confined areas such as sewers, basements, and tanks. Environmental fate and transport of these chemicals and vapors are controlled by chemical/physical processes including volatilization, solubility, sorption to particulate matter, biodegradation, photolysis, hydrolysis, oxidation, bioaccumulation. Systemic toxicity to these gases most commonly occurs after ingestion, inhalation or less commonly, after extensive dermal exposure. These types of exposure can cause nausea, vomiting, hematemesis, dermal and ocular irritation, sedation, dizziness and coma. Respiratory depression and even death can occur in significant exposures. Although it is not possible to completely avoid such incidents, a detailed analysis of facilities vulnerabilities and adequate remedial/safety measures can minimize the effect of these cases.

Medicaments and healthy food take the most important part in human and animal health. The level reached by these products makes necessary these areas to develop as an industry. In other words, it necessitates infrastructures required for mass-productions. The infrastructures of the industrial areas can be used for storage of active and inert ingredients as well as pharmacologically and toxicologically active solvents including acids and alkalis used during the manufacturing process in big amounts. It is known that these chemicals are stored either in a protected or in an unprotected buildings of the industrial establishments which make them an open target for the terrorist attack, sabotage and for wars. Especially, the experience gained during the Golf War and the CB Medical Treatment Congress held in Croatia between 25-31 October, 1998 which highlighted the artillery attack on the refinery in former Yugoslavia lead us to plan this study. In the case of previously mentioned types of attacks on the

pharmaceutical and chemical industries, exposure to the toxic chemicals by inhalation, oral, dermal and ophthalmic routes can cause serious effects to the human health. Therefore, it is thought to be useful to investigate types of risks associated with these events and how the industries are prepared for such cases.

MATERIALS AND METHODS

Pharmaceutical Industry: Pharmaceutical industry is one of the industries likely to be exposed to the above mentioned risks. Therefore, we have prepared a questionnaire consisted of 11 questions. In this questionnaire, the following questions were asked:

- number of the personnel/workers
- whether they produce flammable/explosive chemicals and solvents, if so, what are they types of the chemicals they store
- storage period and conditions
- potential health and environmental effects of toxic liquids and vapors produced from fire and explosion as result of industrial accidents, terrorist attacks, sabotage and earthquake.

Chemical Industry: An investigative report on the soil and ground water quality in the tank area after the earthquake on August 17, 1999 in Yalova, Turkey and summary of the literature will be presented.

RESULTS AND DISCUSSION

Pharmaceutical industry: More detailed investigation on this subject will bring the detailed information on the agenda of the forthcoming meetings. However, it must be pointed out that detailed investigation of this matter is a prerequisite for human health and environmental safety Thus, preparations and precautions on education of personnel/workers and public, safety measures taken or to be taken, and medications to be used for protective and medical treatments purposes can be explored. The results of this questionnaire can be listed as follows:

- Pharmaceutical industries of interest do not produce flammable/explosive chemicals and solvents. Therefore, vast quantities of these chemicals are not stored in their industrial area.
- Flammable/explosive chemicals and solvents are mainly used in the quality control and analytic laboratory work, and are stored in the appropriate part of the industrial area.
- The solvents stored are acetone, ethyl acetate, hexane, diethyl ether, butyl chloride, methanol, isopropyl ether, isopropyl alcohol, and butyl alcohol.
- Depending of the company's consumption, they are stored for a period of 0-3 months.

Although, there is no specific program associated with these matters in the industries, it can be drawn a conclusion that industries can provide answers to some of these cases by using SOPs required by various legislations and ISO standards. Nevertheless, it is important to note that we could not see any specific education program against terrorism, sabotage and natural disasters in the pharmaceutical and chemical industries. .

The results of this questionnaire revealed that depots that are used by the pharmaceutical industries do not have any specific measures for protection, making them vulnerable to the terrorists attacks and sabotage. For example, it is known that the above mentioned solvents can cause mucous membrane irritation, irritation to the eyes, nose, throat, and respiratory tract, dermal irritation, nausea, vomiting and hematemesis, CNS depression ranging from sedation to dizziness to coma. Respiratory depression and even death can occur in significant exposures.

Chemical Industry: In the case of chemical industries, majority of the inputs used in the pharmaceutical industry are the products of chemical industries. This implies that the inputs used in the chemical industries can be more dangerous to the human and animal health and environment. Although Turkey is not a rival country for the basic chemical industry, various industries (dye, textile, fiber, petrochemical, furniture, pigment etc.) are using different types of chemical technologies for their production. Therefore, these industries are required to fulfill the safety measures not only for the war, but also for the terrorism and sabotage. In doing so, they must consider the previous experiences encountered.

Although it is not directly related with the topic in terms of sabotage and terrorism, spillage of toxic chemicals occurs as a result of a natural disaster such as earthquake could present the same degree of danger to the human health and environment. Therefore, we would like to point out acrylonitrile spillage occurred during earthquake on August 17, 1999 in Yalova, Turkey.

Acrylonitrile ($CH_2=CHCN$; MW: 53.06) is an important industrial chemical intermediate used in the production of acrylic and modacrylic fibers and other important chemicals and resins. Five million tons of acrylonitrile is consumed worldwide: 3-million tons are used in ABS plastics, and the remainder in textile industry. It is very soluble in water (at 20° 7.35 parts dissolve in 100 parts water) and highly volatile (100 mmHg at 25°C) with a Henry's Law constant of 0.063 at 25°C.

The largest plant of the acrylic industry in Yalova, Turkey is producing synthetic fiber by acrilonitrile polymerization technology, and has an ISO 14001 environmental management certificate. It also practices very effective applications on environment, human health and technical safety under the Responsible Care program required by the Society of Turkish Chemical Industry. So far, no chemical leakage has been occurred since its establishment in 1969. However, during the earthquake on August 17, 1999, three of the storage tanks have been damaged and approximately 6500 tons of acrylonitrile have been spilled on the soil . Of these, 3000 tons have been recovered and transferred to the other tanks. Although, acrylonitrile is an explosive, flammable and toxic liquid, this unfortunate accident was controlled without any explosion or fire. To explore its effect on the terrestrial life, samples were taken from the sea products, crops and well water in the nearby region and analyzed. The results of these analyses revealed that the effect on plants was limited to the area of 150-200m from the damaged tanks but this effect is short lived. Furthermore, neither the sea products nor the well water were found to be contaminated by acrylonitrile (3).

SUMMARY

To emphasis the importance of this matter, we have decided to present the knowledge obtained from a preliminary questionnaire study on handling, storage and safety of chemicals, and also data derived on this subject. Although it was not possible to investigate these matters in a more detailed manner in a relatively short period, a more detailed investigation with regard to these cases will show pharmaceutical and chemical industries educational and safety measures against terrorism, sabotage, natural disasters as well as chemical and biological warfare.

The example of acrylonitrile can easily be extended to the other chemical industries producing toxic, acidic or basic substances which can be used relatively safely under normal conditions. However, large quantities of these substances can be released to the environment as a result of sabotage, or terrorist attack, and some undesirable effects could persist on terrestrial life and human health. Therefore, extra safety measures must be taken to avoid such incidents as a result of terrorist attack or sabotage.

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KEYWORDS

Risky chemicals, pharmaceutical industry, chemical industry, terrorism, sabotage, earthquake

70. THE ROLE OF POISON INFORMATION CENTERS IN EMERGENCY ASSISTANCE AND PROFESSIONAL/PUBLIC EDUCATION FOR CBW

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INTRODUCTION

The experiences of the last decade have thought two lessons to all. The first was that there were countries that have chemical and biological weapons, and there were other countries that might obtain or produce them. The second was that the ready availability of deadly agents and willingness of extremist organizations to use them introduce a high likelihood that a chemical or biological incident targeting civilians as well as military personnel may take place on any country at any time.

Political and economic changes happening over the past decade also contributed to the CBW market by causing the availability of unemployed weapon specialists and scientists. It has been realized that while it would be more difficult for a country to mass-produce classic CB agents in large quantities without detection, it would be very easy for a country or organized group to develop the technological capabilities to produce very effective biological agents. A legitimate chemical facility can be easily converted for the manufacture of chemical agents, and the dual-use nature of production facilities is even more applicable to the production of biologicals. Furthermore, biotechnology itself seems to be the threat of the future.

On the other hand, various properties of CBW agents make them highly advantageous for the producers and/or users, such as low cost and low technology requirement, limited capability of their detection, extremely frightening image, enormous ability to inflict casualties and severity of their effects. CBW bypasses the tremendous financial outlay required for conventional weapons, and their threat is itself an effective weapon. In fact, they are frequently called as "the poor man's atomic bomb".

PROLIFERATION OF CBW

Although more states than ever signed international agreements to eliminate chemical and biological arms in the second half of the 20th century, yet more are also suspected of developing these weapons despite the treaties. E.g., the Biological Weapons Convention was ratified in 1975, but it did not slow the massive Soviet program for a long time, nor did it prevent the stockpiles of Iraq. According to official reports, over 30 nations either possess or have the ability to develop an offensive chemical weapons capability and over 10 nations either possess or have the ability to develop an offensive biological weapons capability. Hence, the nature of war is changing, the weapons of war have already changed and seem to continue to change and proliferate. And mainly due to state-sponsored terrorism, religious fundamentalism, unemployed and disenchanted weapon specialists and scientists, chemical and biological terrorism seems to continue as a threat to humankind of our time. However, throughout the history, humankind have always been interested in using biological and/or chemical means in destroying enemies, e.g., the use of wells contaminated with infectious agents and arrows with curare has not been infrequent in the early years. But, the interest in the 20th century has increased enormously along with the high level of scientific and technological progresses, although CBW is considered unethical and immoral; and a cruel, unfair and improper use of science. It seems that in no future war will any military be able to ignore them, since they are higher and easier forms of killing!

What would be the solution, then?

SOLUTION: PREPAREDNESS

The lessons of past dictate us that "preparedness" with knowledge and equipment are mandatory to combat with CBW. The likelihood of a chemical or biological "Pearl Harbor" should always be considered by any nations, and to prevent the consequences related bodies including military forces must continue to learn about chemical and biological warfare.

Knowledge/information is essential for the preparedness. Even the thought of chemical or biological warfare terrifies everybody. The fear of an unknown agent that cannot be seen, is not understood is very high compared to conventional weapons. Experiences have showed that the likelihood of CBW causing panic among military personnel decreases when the leaders and troops become better educated regarding these agents. Therefore, by using the statement of General John J. Pershing made after the World War I, it should be emphasized that "the effect is so deadly to the unprepared that, it can never be afforded to neglect the question".

ACCESSEBILITY OF KNOWLEDGE

It is clear that the passive countermeasures, including pretreatment, first aid and casualty handling, therapies, warning and detection, individual protection, collective protection, decontamination, safe devices, and effective use of them for chemical and biological defense significantly reduces the threat to both military forces and civil populations. However, it is also clear that not all the states, especially those closest to the threat are ready enough with the knowledge, equipment and capabilities of their responsible civil bodies, to protect their fellow citizens, and reduce the harm in case of an attack. It is now reported by US sources that it was realized just before the Gulf War that medical personnel were not prepared to provide care to chemical and biological casualties or to function in a contaminated environment. However, before the start of the operation, rapid and intense training programs were applied to prepare medical healthcare providers. In the following years, with the aim to master all relevant aspects of defense against CBW, the US military prepared two handbooks and one comprehensive textbook containing invaluable information that has not been readily accessible before.

It is sad to mention that during those fearful days of the Gulf Crisis, the lack of accessible information was the second serious threat for the civilians of surrounding countries. Fortunately, that era which left the civilians in ignorance for the sake of "secrecy" is over. Thanks to the electronic media and to those views that armed forces as well as civilians, including healthcare providers, can be educated at minimal cost and with great potential benefits.

THE ROLES OF PICs

We still have serious doubts concerning the capability of those nations with shortage of resources but also with CBW suspect neighbors, affording to prepare themselves for an effective civil defense. But we continue to believe that a cooperative effort of both military and civil organizations, and academic institutions can accomplish many things in the presence of accessible information and knowledge. Prevailing attitude of ignorance and fatalism can be changed, and the expression of "out of sight, out of mind" can be stricken out of memories.

It is our opinion that a poison information center (PIC) with its scope, structure and facilities, with its personnel of toxicological background and experience in addition to its possible links with related bodies and contacts with international organizations may relatively readily follow the most recent information and advances in the field of CBW, new chemical agents, biologicals, bioterrorism, toxins, new treatments, new detectors and decontamination capabilities as apply to CB casualties. Then, such a center can disseminate the collected information efficiently to the related bodies.

More generally, the role of a PIC could be in two directions:

- Contribution to civil defense
- Coordination and provision of information and risk assessment for the healthcare providing institutes.

The first task could be accomplished by providing information materials, and teaching and training through short courses. The same would be applied for the second direction covering all aspects of CBW, appropriate first aid, decontamination, antidotes and medical treatments. Furthermore, in case of a crisis, by collecting and evaluating the data concerning the type and nature of the event, the number of victims, the type of injury, the nature of the toxic agents, decontamination of the victims on the scene and estimation of the arrival time of the victims to the hospitals, the healthcare providing facilities can be properly informed in order them to be prepared for effective handling and care of casualties.

ACTIVITIES OF HIZBIM

As a university based Drug and Poison Information Center, HIZBIM (Hacettepe Drug and Poison Information Center) has always been with the aim of establishing such a cooperative and coordinative role between the related bodies that could most probably be involved in mass management and civil defense. However, in addition to before-mentioned existing problems of the country, the devastating earthquakes that we have faced recently caused serious economical and social problems that needed immediate solutions, and affected our programs that planned to be implemented earlier. However, we are now in the process of organizing a training program in cooperation with toxicology, microbiology and infectious diseases departments, and the medical and pharmaceutical associations with the aim to provide information on the various aspects of CBW for civil organizations and healthcare providers. Furthermore, HIZBIM has prepared updated information materials for CBW in different levels by revision of the existing ones that were prepared within the center during the Gulf War. Writing up a comprehensive book, which will be the revised copy of the handbook produced by its personnel during the same period is continuing. It will cover all the aspects of CBW including decontamination, medical treatment and civil defense. Finally, preparations to produce an electronic database on CBW are in the process.

CONCLUSION

If it is not possible to prevent the proliferation and irresponsible use of biological and chemical weapons by either small terrorist groups or by states, then the preparedness of the medical professionals and civilian populations along with the military forces is mandatory in order to limit the qualitative and quantitative consequences. With the continuous developments toward the creation of new weapons on one hand and with the ongoing efforts and advances in the field of medical management on the other hand, continuous education and training, and coordinated planning are necessary for an efficient civil defense. Both past and recent experiences from wars and terrorist attacks from different parts of the world indicate that a wide range of professional categories, such as specific military groups, police forces, civil defense personnel, public transportation systems and medical institutions, could most probably be involved in the mass casualty management. Poison Information Centers and services may well be in the linkage of these different parts and may serve as coordinating reference units with their medical and informational structure, mission and functions.

KEYWORDS

CBW, Civil Defense, Preparedness, and Accessibility of Knowledge, Poison Information Centers

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71. ROLE OF CHOLINESTERASE ACTIVITY IN THE TREATMENT OF ORGANOPHOSPHATE INTOXICATION

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INTRODUCTION

Organophosphate insecticides (OP), one of the anticholinesterase agents, are more toxic and have been responsible for more human deaths than other pesticides. We treated 160 seriously acute intoxicated patients exposed to OP, between 1991 and 1999 in the intensive care unit. 12 patients (7,5 %) died due to complications of ARDS and sepsis.

Namba Classification and serum cholinesterase activity (sChE) are taken into consideration for the diagnosis and management of the treatment.

PATIENTS AND METHODS

We introduce two female patients (ages 18 and 47) exposed to severe OP (Namba IV) poisoning. Each patient was followed daily, according to Namba Classification and sChE activity, as determined with Ellman's spectrophotometric method (range 40-80 U/mL).

Case A: K.T., 18 year old female: 25 % Chlorpyrofos solution ingestion for suicidal purpose

She had Namba Grade IV and sChE was 16,1 U/mL.

After initial doses of atropine and pralidoxime, 4 mg. atropine/day and 1 gr 2-PAM/day were infused for eleven days and stopped. sChE levels increased on the seventh day (27 U/mL), but the day after suddenly decreased (3 U/mL). Improvement of the sChE and with that, her Namba grade, was observed on later days. She was discharged on the fifteenth day with an sChE level of 18,2 U/mL and Namba grade I. Seven days afterwards, in a control examination, her sChE was found to be 31,5 U/mL. This is shown in Figure 1.

Case B: E.G, 47 year old female: Unkown OP insecticide ingestion for suicidal purpose

She had Namba Grade IV and sChE was 26,6 U/mL.

After initial doses of boluses atropine and 2-PAM, 1mg Atropine infusion/day was started and this dose was gradually increased up to 24 mg on the ninth day. In addition, 2 g 2-PAM were also infused during these days. Both of the drugs were ceased on the ninth day, due to improvement of her grade and enzyme level (Namba Grade II. sChE 40 U/mL.). However, she deteriorated clinically and the sChE decreased to 10 U/mL on the fourteenth day. We started 0,5 mg Atropine/day and 0,5 gr 2-PAM/day infusion again and continued until 28th day after admission. Patient was discharged with Namba Grade I and 32,2 U/mL. Ten days later, when she came in for a control exam. her sChE level was 35 U/mL. This is shown in Figure 2.

NAMBA CLASSIFICATION (1)

This clinical classification is explained below.

LATENT POISONING: **Namba I**

No clinical manifestation, sChE inhibited 10-50 %.

Treatment unnecessary, prognosis good.

MILD POISONING: **Namba II**

Fatigue, headache, nausea and vomiting, abdominal pain, diarrhea, excessive sweating and salivation, sChe inhibited 20-50 %.

Treatment oxime and atropine, Prognosis good.

MODERATE POISONING: **Namba III**

Generalized weakness, difficulty in speech, muscular fasciculations, miosis, sChE 10-20 % of normal.

Treatment oxime, atropine, mechanical ventilation, prognosis good.

SEVERE POISONING: **Namba IV**

Unconsciousness, pin-point pupils and loss of light reflex, muscular fasciculations, secretions from the mouth and nose, rales in the lungs, respiratory difficulty, sChE less than 10%

Treatment oxime, atropine, mechanical ventilation, prognosis fatal, if not treated

OP INTOXICATION TREATMENT SCHEDULE

From our experiences, the following treatment schedule is recommended:

INITIAL DOSES

Atropine: 8 mg i.v. bolus

Pralidoxime: 1 g i.v. bolus

MAINTENANCE

Atropine: 1-4 mg infusion/day

Pralidoxime: 0,5-2 g infusion/day

Benzodiazepines for convulsions

EVALUATION

sChe Activity/day

Namba Evaluation/day

ADDITIONAL

Mechanical Ventilation, Enteral or parenteral feeding

DISCUSSION

Atropine and oximes are cornerstones of the treatment for OP poisoning. When given together, they act synergistically against the signs and symptoms. It is a very interesting point that the sChE decreased and the clinical status of each patient deteriorated in the middle of the treatment. This can be related to redistribution of the lipophilic substance or oxon metabolites of Chlorpyrofos, which are 400 times more active as a cholinesterase inhibitor than the parent compound.(2). Recovery took a short time, five days, and was easier when the treatment was continuous as in Case A. However a longer period, fifteen days, was required, when the treatment ceased as in Case B.

CONCLUSION

Namba Classification and sChE level are the main criteria for the diagnosis and the severity of OP poisoning.

Treatment can be maintained with their guidance, but the kind of substance and metabolites can change the properties of recovery period. The clinical situation is much more important than the sChE levels in deciding the cessation of antidotal therapy, because it will take at least 4 to 6 weeks to return to normal values of sChE.

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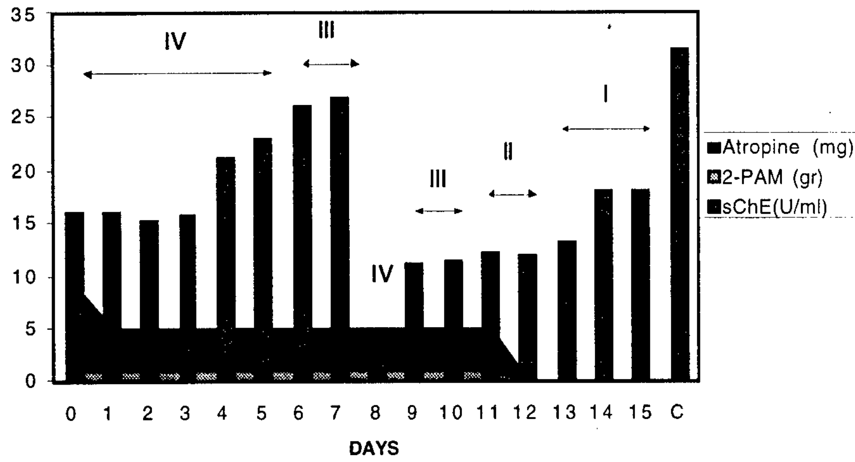
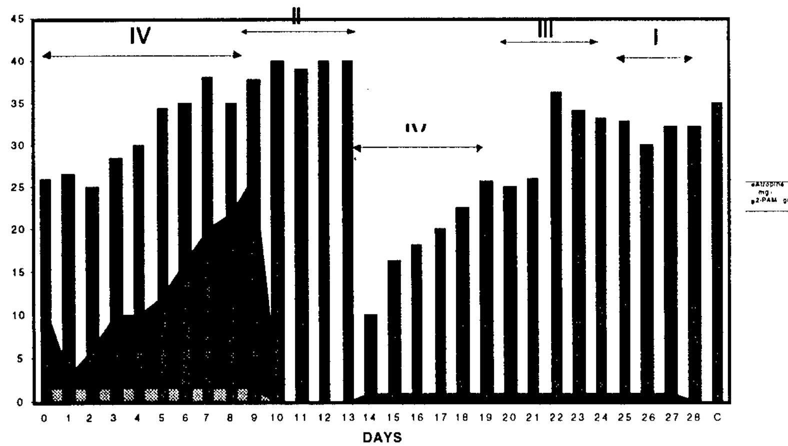
FIGURES AND TABLES

GRAPHIC I



CASE A: K.T., 18 year old female, 25%Chlorpyrophos ingestion



CASE B: E.G, 47 year old female, unknown OP ingestion

72. KINETICS OF NERVE AGENT HYDROLYSIS BY A HUMAN PLASMA ENZYME

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ABSTRACT

In the search for new nerve agent scavengers the organophosphorus acid anhydride hydrolase (OPAA hydrolase) enzymes from human plasma (paraoxonase 1, PON1) have emerged as very promising candidates. Although the turnover rates of these enzymes are not believed to be high enough to confer a great amount of protection against nerve agents, it is probable that those rates can be improved by protein engineering once high-resolution crystal structures are available. It was previously shown that both known isozymes of PON1 are able to hydrolyze the toxic isomers of soman, sarin and, to a lesser extent, tabun. We now have shown that in spite of the rather high Km values of these enzymes with these agents, sarin and soman are hydrolyzed quite rapidly even at submicromolar concentrations. We have also demonstrated that the enzyme is able to catalyze the hydrolysis of VX. In contrast to the reactions with soman and sarin, both of which produced good Lineweaver-Burk plots from which Km and Vmax could be calculated, this reaction appears not to follow Michaelis-Menten kinetics; the rate of reaction continues to increase with substrate concentration at least up to 3.2 mM VX at which point the hydrolysis rate is 0.1 μmole per minute per mg enzyme, a value that is comparable to the value of 0.16 μmole per minute per mg enzyme reported for the hydrolysis of VX by the Co^{++} form of the OPAA Hydrolase from Pseudomonas diminuta, the most efficient VX-hydrolyzing enzyme reported previously.

INTRODUCTION

In the search for a suitable *in vivo* scavenger to protect personnel against nerve agent intoxication, we have considered many enzymes. In general, those that are available in nature are not suitable catalytic scavengers because they bind the nerve agents poorly and their hydrolytic turnover rates are too slow. We have attempted to remedy this situation by engineering enzymes that are inhibited by the organophosphorus anticholinesterases in a way that they behave as organophosphorus acid anhydride hydrolases (OPAH), that is, they catalyze the hydrolysis of nerve agents. We began with that strategy because the crystal structure and DNA sequence were available for acetylcholinesterase (AChE) [1] and from that crystal structure a model could be constructed of butyrylcholinesterase (BuChE) [2] and carboxylesterase (CaE) [3], whereas the folded structures of OPAH enzymes were (and remain) unknown. Unfortunately, enzyme-engineering experiments have been only partially successful to this point in time and though the desired activity was produced the required turnover rates have not been approached [4, 5, 6]. This leads us to pursue other avenues for the discovery of a catalytic scavenger. One promising candidate is the in human plasma OPAH, PON1, a member of a family of esterases associated with high-density lipoprotein. Their natural function has been shown to include the hydrolysis of homocysteine thiolactone, a protective mechanism against protein N-homocysteinylation [7]. Mice in which this gene has been knocked out are highly sensitive to organophosphorus insecticides and are susceptible to atherosclerosis [8]. It has a molecular weight of about 35 kD, similar to that of OPAH from Ps. diminuta, the crystal structure of which has been solved [9]. However, there appears to be no homology between the two classes of enzymes. The enzymes differ in metal ion requirements (PON1 requires Ca++, the Pseudomonas enzyme requires Zn++ or Co++). Although there are three cysteine residues in each protein their positions in the sequence are completely different.

Several years ago we studied the hydrolysis of soman, sarin and tabun by human PON1 [10]. The results are shown in Table 1.

In humans PON1 exists as two phenotypes with either Gln (type Q) or Arg (type R) at position 192 in the amino acid sequence. As can be seen in the Table, both soman and sarin are hydrolyzed by either form, but with a high KM, though the Q variant seems to be significantly faster. Tabun was also hydrolyzed measurably, but at a much slower rate (data not shown). It was of interest to determine whether VX hydrolysis would also be catalyzed by this enzyme and whether this enzyme activity is of practical significance at the low concentrations attained in an exposure of a few times the LD50.

MATERIALS AND METHODS

PON1: Drs. Masson and Josse have been involved in an intense study of the PON enzymes recently and have produced highly purified preparations, which they provided to us at Edgewood for study of the kinetics of VX hydrolysis. They have also produced several mutants in cell culture media that they provided for some of the low concentration studies.

VX Hydrolysis: The hydrolysis of VX is conveniently measured by following colorimetrically the liberation of the thiocholine analog using 5,5'-dithiobis-(2-nitrobenzoic acid).

By utilizing a microtiter plate reader with a 405nm filter, a series of up to 12 VX concentrations were measured simultaneously in triplicate; thus all reactions in any given run proceed under identical conditions, an ideal arrangement for kinetics measurements. Beginning with the highest concentration of VX that may be handled in our laboratory under surety regulations, 3.44 mM, a series of dilutions was made using a mixture of 90% 1.1M NaCl/2.2mM CaCl2 and 10% 0.2M

Tris buffer (either pH 7.4 or pH 8.5) as a dilutant: Generally the dilutions were serially 4:5. To each concentration of VX (1 mL) was added 20 uL of 0.02M DTNB, the solutions were mixed thoroughly and 100 uL of each concentration was admitted to a row of wells in a microtiter plate. The final VX concentrations ranged from 3.21 down to 0.28 mM. Three microliters of the appropriate enzyme solution was added to each well and the plate was read for 20 min. One row was kept without enzyme to measure background hydrolysis, if any. The hydrolysis rate in each well was calculated from the slope of the line measured between 0 and 20 minutes, a total of 41 points. Each enzyme sample was run in triplicate and at two pH values.

G Agent Hydrolysis, millimolar range: In order to compare the VX hydrolysis rates with those of G agents the hydrolysis of soman was measured under comparable conditions. Soman was diluted serially beginning at 11mM and then each of those dilutions was brought to the same ionic strength by the addition of an equal volume of 1M NaCl/2 mM CaCl2, creating a concentration range from 5.45 to 0.16 mM. Detection of hydrolysis rate was by pH stat. All data were normalized to nmol agent hydrolyzed per minute per µL enzyme used so that they could be compared.

Agent hydrolysis, micromolar range: First, a solution of cholinesterase (either acetyl or butyryl) was prepared such that when 10 microliters of 1 micromolar agent was added to a 50 microliter aliquot the activity was just shy of being completely inhibited. Then, to 100 microliters of a solution of the enzyme to be tested , equilibrated to 30 deg., was added a solution of the agent to be tested such that the final concentration was exactly one micromolar and mixed on a vortex mixer. As quickly as possible (usually 10-12 seconds) a 10 microliter sample of the mixture was transferred to 50 microliters of the cholinesterase solution, mixed and held at room temperature for 10 minutes to allow reaction between the cholinesterase and the residual agent. Similar samples were taken at predetermined times after being mixed and treated in the same manner. After the ten-minute incubation period the cholinesterase activity of the sample was determined and the concentration of residual agent calculated. The linear portion of plots of residual agent concentration versus time provided initial rates of agent hydrolysis. Minor variations of this procedure permitted determination of agent hydrolysis rates between 0.25 and 10 micromolar initial concentrations.

RESULTS

The VX hydrolysis results at millimolar concentrations are shown in Figure. 2. The black circle is a determination at a single substrate concentration (1 millimolar) with the OPAH from Pseudomonas diminuta for comparison: this enzyme is reported to have the highest turnover rate for VX of any enzyme tested so far. It is noted that VX hydrolysis by the human PON is faster at pH 8.5 than at 7.4. This is not unexpected; with the G agents and insecticides rates of hydrolysis are faster at higher pH. However, it is surprising that the human enzyme is faster at both pH values than the rabbit enzyme is at the higher pH. It should be pointed out that the enzyme solutions used for the VX data are the same as those for the soman data in Figure. 3. The rabbit enzyme hydrolyzes soman about 10 times as fast as the human enzyme while VX is hydrolyzed much more slowly. It is also interesting to note that the Ps. Diminuta OPAH is much slower with GD than the rabbit PON, but much faster with VX than either PON.

The results of studies at low micromolar substrate concentrations are not yet as quantitative as those at the higher concentrations, though they are, perhaps, more pertinent. It is clear that both human PON variants are able rapidly to hydrolyze the G agents tested, with very little difference at these concentrations. The Q variant (the most abundant one) is also able to catalyze the hydrolysis of VX. The ability of the R variant to hydrolyze VX has not yet been conclusively demonstrated; pure R type PON is difficult to obtain from natural sources. Recombinant Q192R has been made by Dr. Josse and will be available for future testing.

CONCLUSION

We have shown that PON1, the OPAH from human plasma, is able to catalyze the hydrolysis of VX at a rate comparable to the OPAH from Pseudomonas diminuta, the best enzyme for that purpose yet described. Additionally, we have shown that nerve agents are destroyed by PON1 even at the low concentrations attained in blood at lower levels of exposure. Furthermore, since this enzyme obviously was not designed to catalyze the hydrolysis of nerve agents, it should be amenable to enzyme-engineering techniques to improve that activity. Because it is a natural plasma enzyme it should be compatible with the human organism and expected to be tolerated well if exogenously

augmented. Efforts are in progress to obtain diffracting crystals of PON1 so that a crystal structure can be obtained that we hope will enable us, and others, to design possible mutants with improved nerve agent binding and faster hydrolysis rates that can be used as practical nerve agent scavengers.

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FIGURES AND TABLES

Figure 1. The hydrolysis of VX is conveniently measured by following colorimetrically the liberation of the thiocholine analog using 5,5'-dithiobis-(2-nitrobenzoic acid).

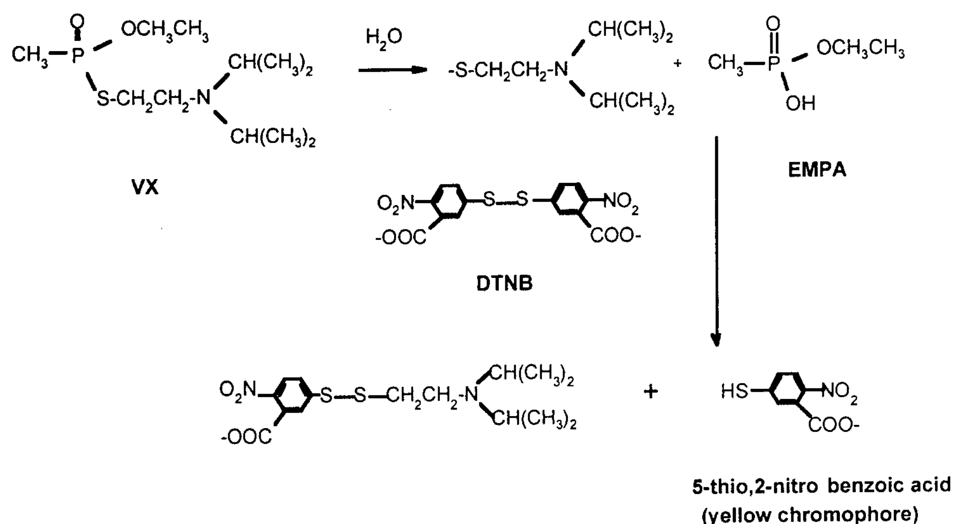

Figure 2. The VX hydrolysis results at millimolar concentrations. The black circle is a determination at a single substrate concentration (1 millimolar) with the OPAH from Pseudomonas diminuta for comparison

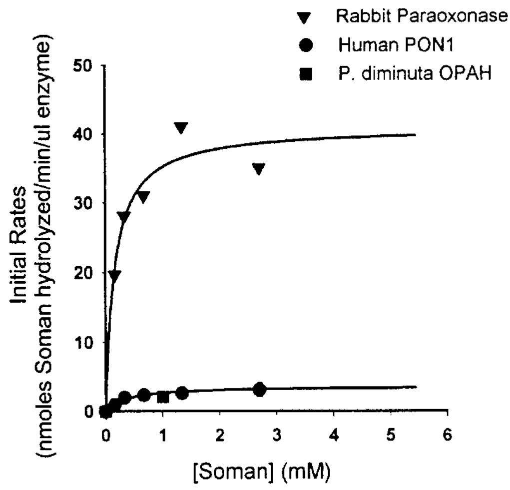

Figure 3. Hydrolysis of VX.

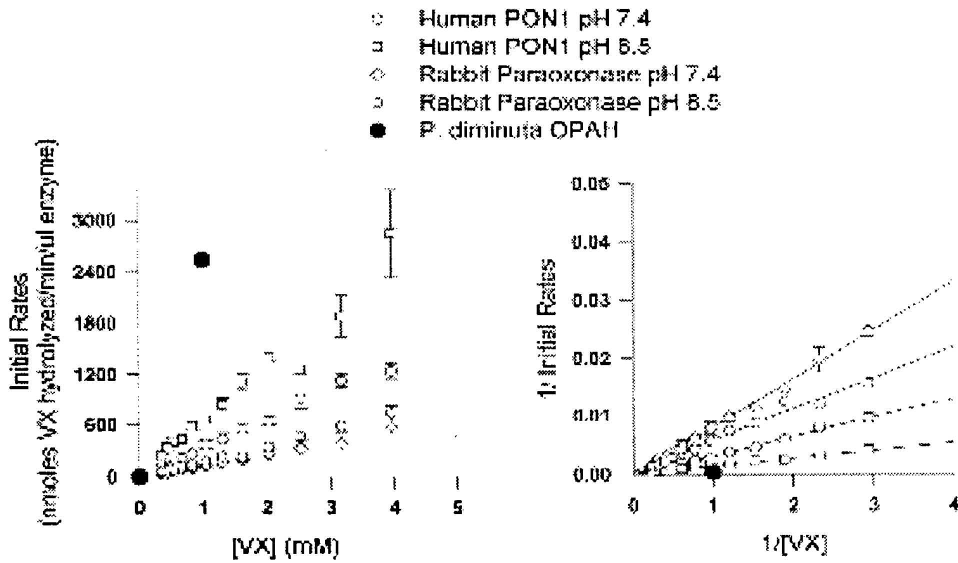

Table 1. Hydrolysis of Sarin and Soman by Plasma OPAH

Enzyme	Sarin		Soman	
	Km	Vmax	Km	Vmax
Hu 192Q	0.21	69	0.41	82
Hu 192R	0.31	21	0.25	31
Heterozygous	0.15	24	0.39	36

Units for Vmax = micromoles/minute/milligram protein

73. WIDE-RANGE APPLICATION OF *ALTEROMONAS* PROLIDASE FOR DECONTAMINATION OF G-TYPE CHEMICAL NERVE AGENTS

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ABSTRACT

Organophosphorus acid anhydrolases (OPAA: EC 3.1.8.2) are capable of catalytically hydrolyzing a wide range of organophosphorus compounds, including the fluoride containing chemical nerve agents such as soman and sarin. The genes encoding OPAAs have been cloned and sequenced from two species, *Alteromonas sp.*JD6.5 and *A. haloplanktis*. Sequence and biochemical analysis of the cloned genes have established OPAA to be prolidases (E.C. 3.4.13.9), a type of dipeptidase hydrolyzing X-proline bonds. The development of recombinant clone XL1 (pTCJS-4) producing large quantities of active G-agent degrading enzyme, *A. sp.*JD6.5 prolidase, offers a real potential for large-scale production of the enzyme. With this enzyme, a simple and safe enzyme-based decontamination system that provides long-term stability and greatly reduced logistical burden was achieved. Formulation of the recombinant enzyme in different matrices provides opportunity for chemical agent decontamination, personnel protection, and detection. Such matrices include fire-fighting foams/sprays, environmentally safe detergents and degreasers. Retention of hydrolytic activity for extended period of time in lyophilized form allows enzyme use with existing spray or fire-fighting equipment. Appreciable retention of prolidase activity in various matrices, cross-linked prolidase enzyme crystals (CLEC), cellulose binding domain (CBD)-linked form, polyurethane foam conjugated form, offers wide-range applications. The prolidase enzyme is also stable in commercial laundry detergent "Tide-Free", and in "Protectall" skin lotion.

INTRODUCTION

Enzymes detoxifying the highly toxic acetylcholinesterase inhibitors are generally classified as organophosphorus acid anhydrolases (OPAA; EC 3.1.8.2), and have been found in both prokaryotes and eukaryotes (1,2). Of the microorganisms investigated thus far, several isolated halophilic bacterial strains including a number of *Alteromonas* species were found to contain high levels of OPAA activity (2-4). Diisopropyl flurophosphate (DFP) which has been routinely used in research as a serine protease inhibitor, is a common substrate used to screen for OPAA activity. The OPAA enzymes are capable of catalytically hydrolyzing a wide variety of organophosphorus compounds (OPs), including the fluoride (F^-) containing chemical warfare (CW) nerve agents such as soman (GD: *O*-pinacolyl methylphosphonofluoridate), sarin (GB; *O*-isopropyl methylphosphonofluoridate), GF (*O*-cyclohexyl methylphosphonofluoridate), and cyanide containing tabun (GA: ethyl *N,N*-dimethylphosphoramidocyanidate). Recently, the genes encoding OPAAs have been cloned and sequenced from two species, *A. sp.*JD6.5 and *A. haloplanktis* (5,6). Sequence and biochemical analysis of the cloned enzymes have established these OPAAs to be prolidases (E.C. 3.4.13.9), a type of dipeptidase hydrolyzing dipeptides with a prolyl residue at the carboxyl terminal position. The prolidase has been shown to play an important role in cellular dipeptide metabolism in humans.

Previously, a parathion-degrading enzyme, organophosphorus hydrolase (OPH) from *Pseddomonas diminuta* MG and *Flavobacterium* species ATCC 27551, was also found to have a significant G-type nerve agent hydrolyzing activity (7,8). OPH is the only well characterized enzyme known to catalytically hydrolyze VX. However, no sequence homology was found between *Alteromonas* prolidase and OPH.

The currently fielded decontamination solutions, DS2 and bleach, are corrosive in nature and result in hazardous waste. Because of their corrosivity, such solutions cannot be used on sensitive equipment or for personnel decontamination. The use of non-toxic enzymes has considerable potential for situations involving decontamination of sensitive equipment, vehicles, large fixed sites, cleanup operations resulting from a possible terrorist incident, and chemical agent handling during demilitarization and storage. Enzyme-mediated decontamination is non-toxic, non-corrosive, and environmentally compatible. The dry form of enzyme should also result in a significantly reduced logistic burden involved in storage and transportation, perhaps by as much as 50-fold. In this report, the inclusion of enzyme into different matrices and the feasibility of these matrices for decontamination, protection, and detection were evaluated.

MATERIALS AND METHODS

Materials

*Alteromonas sp.*JD6.5 was isolated from Grantsville Warm Springs, Utah, as described previously (3). *A. haloplanktis* (ATCC 23821) and *A. undina* was obtained from American Type Culture Collection (ATCC), Rockville, Maryland. The purification of prolidases from native *A. undina* (ATCC 29660) cells was performed by procedure similar to those described earlier (4). Organophosphorus hydrolase was prepared from XL1 (pVSEOP7) (unpublished work, Rastogi, Cheng, and DeFrank). The construct was prepared by first amplifying the OPH gene using pJK33 (courtesy Walter Mulbry, USDA, Beltsville, MD) as target DNA, and then sub-cloning it into pSE420 cloning vector (Invitrogen, CA). Most of the routine chemicals used in the report were purchased from either Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA).

Enzymatic Assays

Enzyme activity measurements, using DFP as a substrate, were determined by monitoring F^- release with an ion-specific electrode as described previously (3). The reaction mixture consisted of 50 mM $(NH_4)_2CO_3$, 0.1 mM $MnCl_2$, 3 mM DFP, and 0.3-0.4 U enzyme or enzyme matrices in a total volume of 2.5 ml. One unit (U) of enzyme catalyzes the release of 1.0 μmole of F^- per minute at 25°C. Specific activity is expressed as U per mg of protein. Protein concentrations were determined using Coomassie protein assay reagent (Pierce, IL) with bovine serum albumin to generate a standard curve.

Growth of Recombinant Cell Lines and Analysis of the Expressed Enzyme

The plasmid (pTCJS4) carrying *A. sp.*JD6.5 prolidase gene (9,10) was transformed into different *E. coli* strains. The recombinant cells were grown at 30°C in flask using Luria-Bertani (LB) complex media. The media was supplemented with 0.1 mM $MnCl_2$. The cells were induced with 0.6 mM isopropyl-β-D-thiogalactoside (IPTG) at early-mid-log phase (A_{600nm}= ~0.5).

For analysis of the expressed enzyme, 1-ml cells was collected at the time of harvest by centrifugation and resuspended in 2x SDS-PAGE sample buffer [0.125 M Tris, pH 6.8, 4% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), 10% glycerol and 0.025% bromophenol blue]. The samples were lysed by incubating at 100°C for 5 minutes, and then electrophoresced through a 10% SDS-polyacrylamide gel at 100 mA.

Preparation of Freeze-dried Recombinant Enzyme

The recombinant cell lines (*Alteromonas sp.*JD6.5 and *A. haloplanktis*) were grown as described above. After IPTG induction for 5-6 hours, cells were harvested, resuspended in 10 BM buffer (10 mM Bis-tris propane, pH 7.2, containing 0.1 mM $MnCl_2$ and 0.1 mM DTT), and lysed by passing through French Press cell. After removal of the cell debris by centrifugation, the cell-free lysate was subjected to $(NH_4)_2SO_4$ fractionation, and Q-Sepharose chromatography as described previously (9,10). The purified enzyme sample was then mixed with trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) to a final concentration of 250 mM and lyophilized to dryness (11).

Subcloning and Preparation of Prolidase-Cellulose Binding Domain (CBD) Fusion Protein

The *A. sp.*JD6.5 prolidase gene with N-terminal Met codon and the translation termination codon was obtained from pTCJS4 (9) by PCR subcloning using two synthetic primers; 5'-primer (5'-GGGTGATTCATGAATAAATTAGCGG-3') and 3' primer (5'-GGAATTCTACATGAGCCCAGCAG-3'). The amplified gene fragment cleaved with *Rca*I and *Eco*RI was ligated to the *Nco*I and *Eco*RI sites of the pET34DNA, the CBD expression vector from Novagen (Madison, WI). After ligation and transformation into *E. coli* BL21, the recombinant cell line induced by IPTG, was grown to mid-log phase at 30°C. Batch purification of enzyme-CBD fusion protein from cell lysate was performed with Novagen's CBind Resins following manufacturer's procedures. The cotton fabrics, cotton ball (ACME Cotton Products, Co., Inc., Valley Stream, NY.) and cheesecloth (American Fiber and Finishing Inc., Colrain, MA), were soaked in prolidase-CBD fusion protein for 10 min at room temperature and then rinsed with 1% ammonium carbonate solution. After air drying at room temperature, a piece of the fabric was tested for DFP hydrolyzing activity.

RESULTS AND DISCUSSION

The enzymes from different *Alteromonas* species are similar in their biochemical and catalytic properties, and functionally related to X-Pro dipeptidases with a broad range of substrate specificity (5,6). Besides cleaving the C-N bond of X-Pro dipeptides, *A.* prolidases catalyze hydrolysis of a wide range of OPs with P-F, P-O, and P-CN bonds (6), including DFP, soman (GD), sarin (GB), tabun (GA), and paraoxon. As shown in Table 1, the *A.* prolidases from various species exhibit various degrees of enzyme activity against GB, GD and GF, the highly toxic chemical nerve agents. In contrast to OPH, no VX hydrolyzing activity was observed for *Alteromonas* prolidases. Activity of the enzyme on these OPs may be due to the fortuitous similarity of these compounds on size, shape and surface charge to the X-Pro dipeptides.

As reported previously, the DH5α cell line carrying pTCJS4 was shown to produce high levels of *A. sp.*JD6.5 prolidase enzyme (9,10). Recently, we have compared the growth condition and expression level of the enzyme in

other *E. coli* strains, BL21 and XL1, containing pTCJS4. The specific activity of these recombinant cells increased sharply 2 hours after IPTG induction and reached maximum around 5-6 hours after induction. The highest specific activity was observed in lysate from XL1 recombinant cells. Total enzyme recovered from XL1 cells is 32 and 49% higher than that recovered from DH5α and BL21 cells, respectively. After 5 h IPTG induction, the prolidase enzyme in XL1 cells was ~50% of total cell protein (corresponding to ~250 mg/L of culture). The recombinant enzyme has the same molecular weight as the purified enzyme from native cells. The results from the expression level of the enzyme suggest that XL1 is the most suitable host strain for optimum production of the *Altermonas* prolidase.

The full potential of enzyme decontamination technology can only be recognized if large quantity of enzyme can only be produced cheaply. On-going bioreactor fermentations with recombinant cells (XL1/pTCJS4) in our laboratory have resulted in higher recovery and yield (1 g/L) of prolidase enzyme (unpublished results, Kim, Cheng, Rastogi, DeFrank). Compared to shaker flask, the increase in enzyme yield was brought about by corresponding increase in cell mass recovery. Current fermentation protocol has resulted in a high level of enzyme production corresponding to 30-40% of the total cell protein, confirmed both by SDS-PAGE analysis and activity determination of the enzyme (unpublished results, Kim, Cheng, Rastogi, DeFrank). With the availability of fermentors ranging from 30L to 1,500L capacity in Bioprocess Engineering Facility at the US Army Edgewood Chemical Biological Center, large-scale production of *Altermonas* prolidase is now achievable with this recombinant clone. Availability of *Altermonas* prolidase in kilogram quantity is a key pre-requisite for development of a safe and non-corrosive decontamination system.

As reported previously (11), the enzyme can be packaged in a lyophilized form (Figure 1) and can be reconstituted as needed with any available liquid. A number of matrices such as water-based foams and wetting agents for use as enzyme-based decontaminants were examined (9,10). Several biodegradable wetting agents such as "Cold Fire" and "Odor Seal" (FireFreeze Worldwide Inc., NJ), were found to enhance or stabilize enzyme activity. "Cold Fire" effectively and safely suppresses fire, where "Odor Seal" is an environmentally safe odor eliminator. All these agents are biosurfactant-based and biodegradable in nature. The incorporation of such matrices in the enzyme decontamination system not only provides a medium to encapsulate the CW agents but also assist in the solubilization of the hydrophobic CW agents for enzyme action. Foam systems offer many advantages for decontamination of nerve agents in large areas. Interestingly, the enzyme also retained full catalytic activity in commercial laundry detergent "Tide Free" (Procter & Gamble, OH) and "Protectall" skin cream (J.G. Worldwide Medical, NJ). Such active enzyme matrices provide a useful means for protective clothing clean up and wound healing.

In collaboration with researchers in other government agency, academia, and industry, the recombinant *A. sp.*JD6.5 prolidase has been incorporated into various matrices for protection against and detection of chemical G-agents (Table 2). The application of immobilized, catalytically active enzyme to detoxify nerve agents on a variety of surfaces including protective clothing, filters in gas masks, or skin, is being investigated in our laboratory. Since cellulose is an integral component of various cotton fabrics including filters, sponges, cotton, and gauze, the enzyme could be immobilized via coupling of the enzyme to cellulose binding domain (CBD) protein. *A. sp.*JD6.5 prolidase gene was cloned in CBD expression vector, pET34DNA. The recombinant construct generated a recombinant fusion protein (see "Materials and Methods") which is 204 amino acid longer than *A.* prolidase (Figure 2). Based on K_{cat}, approximately 60% of the enzyme activity (~650 U/mg) in the CBD-enzyme fusion protein was retained (Table 2). Loss of activity could be attributed to improper folding of the fusion enzyme. The enzyme activity of fusion protein is quite stable over 3 months period. In addition, the specific activity of cellulose bound CBD-enzyme has been determined (Figure 3). The CBD-enzyme appears to bind to cheesecloth better than cotton. After repeated use, the CBD-prolidase retained ~70% enzyme activity, compared to only ~14% activity in cotton ball. The inability of the CBD-enzyme to bind to cotton may be due to pretreatment of cotton with either dyes or fire retardant chemicals during manufacturer's processing.

Another novel approach for enhancing enzyme activity and stability is by generation of a highly efficient enzyme nanoencapsulation. Through Dr. Ray Yin, US Army Research Laboratory, a number of well-defined, stable, water soluble, non-toxic dendritic polymers was synthesized. The three-dimensional and spherical tree-like dendrimers are capable of nanoencapsulating *A.* prolidase inside their structure. Testing the DFP hydrolyzing activity of the encapsulated enzymes in one of the dendrimers demonstrated that the encapsulated enzymes are not only stable but also improve the catalytic performance of the enzyme (Table 2). Compared to enzyme alone, the activity of encapsulated enzymes was increased by 10-15% at various temperature and pH for a long period of time. Most interestingly, the stability of enzyme activity in dendrimer-enzyme conjugate increased over 100% in organic solvents, such as 20% of methanol or acetone, but not in chloroform. This opens a wide variety of application possibilities for use of immobilized enzyme in areas such as sensitive equipment decontamination, coating protective clothing against agents, and incorporation into skin cream/lotion for wound healing.

The linkage of various enzymes as cross-linked enzyme crystals (CLECs) and polyurethane foam has been shown to provide enzymes with remarkable temperature, pH, and organic solvent stability (12). Formation of enzyme CLECs was achieved following reaction with a bifunctional agent such as glutaraldehyde. The immobilization of enzyme within polyurethane sponges was prepared by chemically incorporated enzyme within polyurethane matrices during synthesis. Through the research collaboration (Table 2), the *A. sp*JD6.5 prolidase in CLEC and polyurethane foam have been prepared and tested against DFP hydrolyzing activity. Preliminary results illustrated that the specific activity of the enzyme were greatly reduced in both CLEC (150-200 U/mg) and polyurethane foam (100-150 U/mg). Nonetheless, considerable enzyme activity was retained in these matrices. The enzyme was stable for over six-month period. The stability and re-usability of immobilized enzyme in such matrices provide a valuable tool for equipment decontamination and personnel protection.

Alteromonas prolidase can also prophylactically protect the mice to the same degree as 2-PAM or atropine, the commonly use antidote, administrated by the intraperitoneal route. When the liposome encapsulated enzyme was combined with pyridinium-2-aldoxime-N-methyliodide (2-PAM) or atropine, almost ten-fold increase in the protection against lethal effects of DFP was observed (Petrikovoics *et al*; see this Proceedings). In addition, effort is now underway using silica gel beads coated with *A.* prolidase in development of biosensor for detection of DFP and other simulants.

CONCLUSIONS

The development of recombinant clone XL1 (pTCJS-4), offers a real potential for large-scale production of the *A. sp.*JD6.5 prolidase, an enzyme with high level of activity against chemical G-agents. Although the enzyme activity in different matrices varies considerably, results have been very promising. The inclusion of enzyme into different matrices that are simple and safe for users, equipment, and the environment, offers a valuable tool for wide-range application of *A. sp.*JD6.5 prolidase. In addition to decontamination applications, the enzyme has also been shown to be potentially useful for protection, surface clean up, and detection. Currently, further wide-range applications are being extensively explored.

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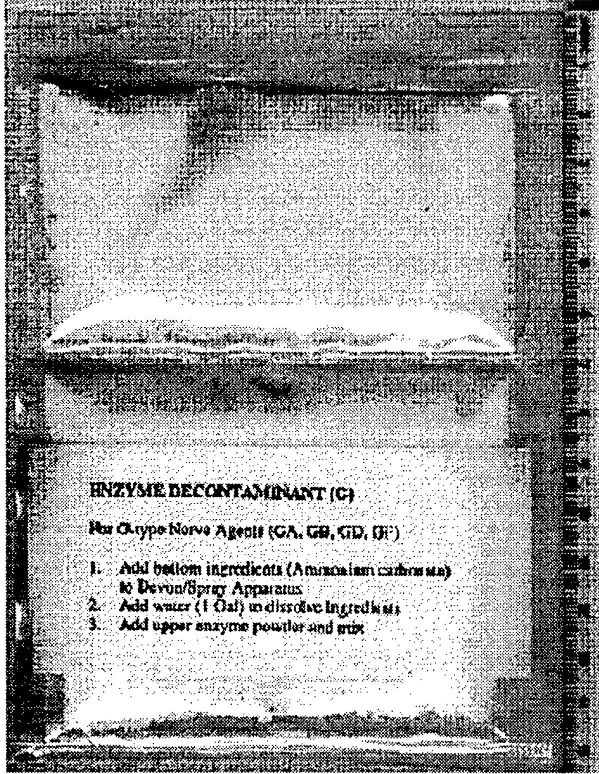
KEY WORDS: Organophosphorus acid anhydrolase, *Alteromonas* prolidase, G-type chemical nerve agents, decontamination and protection.

FIGURES AND TABLES

Figure 1. A prototype of enzyme decontaminant. The package is for one gallon of decontaminant solution. The package contains dried enzyme (upper compartment) and reaction component, ammonium carbonate and $MnCl_2$ (lower compartment).

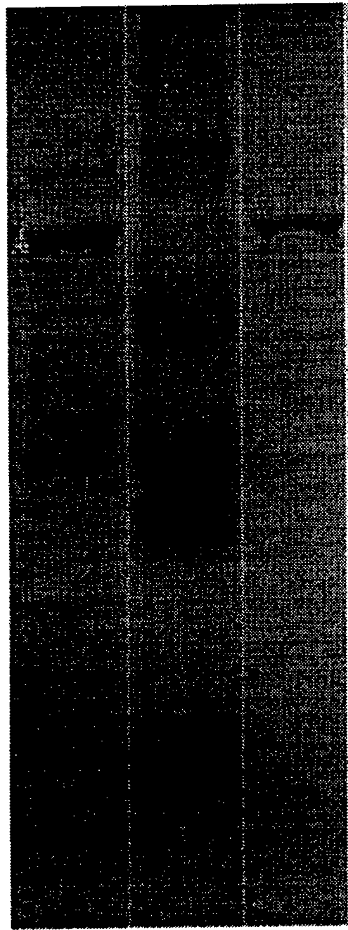


ENZYME DECONTAMINANT (G)

For G-type Nerve Agents (GA, GB, GD, GF)

1. Add bottom ingredients (Ammonium carbonate) to Decon/Spray Apparatus
2. Add water (1Gal) to dissolve Ingredients
3. Add upper enzyme powder and mix

Figure 2. SDS-PAGE Analysis of *A. sp*JD6.5 prolidase-CBD fusion protein.



Crusde Lysate	MW Marker	Pure Enzyme-CBD

Figure 3. Performance of CBD-prolidase immobilized to fabrics

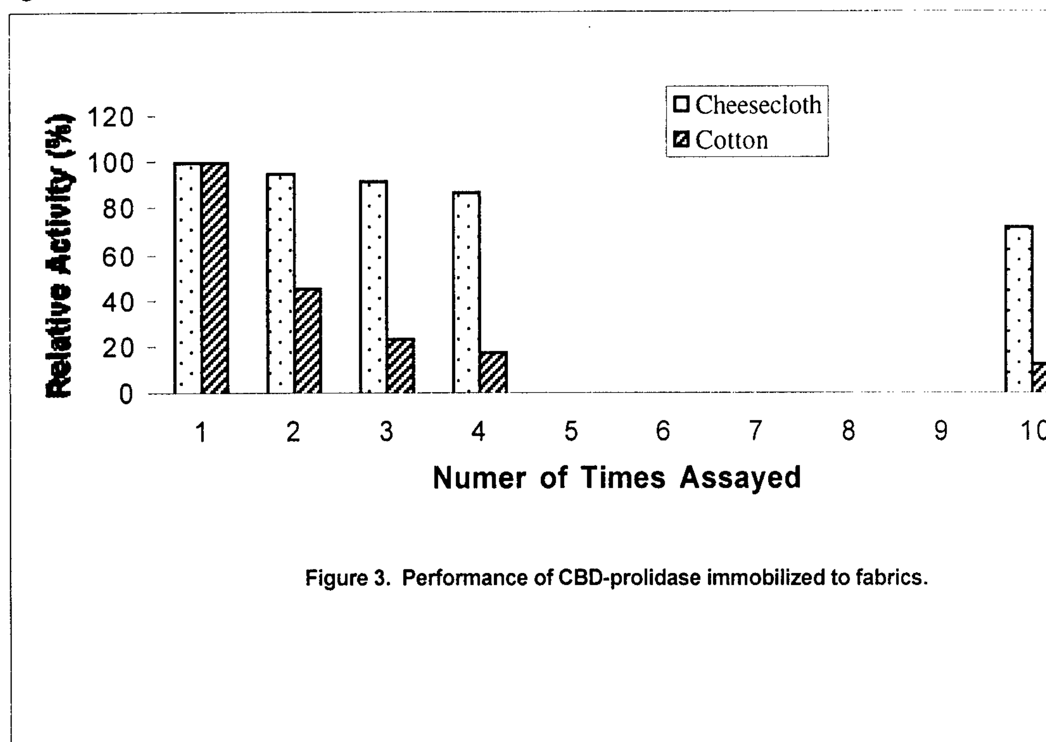


Figure 3. Performance of CBD-prolidase immobilized to fabrics.

Table 1. Comparison of various enzyme activities against DFP and chemical nerve agents

Enzyme Source			K_{cat}	(sec^{-1})		
	DFP	GA	GB	GD	GF	VX
P. diminuta (OPH)	465	N/A	56	5	N/A	0.3
*A. sp*JD6.5	1650	85	611	3145	1650	0
A. haloplanktis	575	113	257	1389	269	0
A. undina	1239	292	376	2496	1586	0

N/A: not available.

Table 2. Applications of *A. sp.*JD6.5 prolidase in various formulation for protection and detection against chemical G-agents

Application	Matrics	Investigator/Collaborator	Specific Activity (U/mg)*
Protection	Cellulose binding domain (CBD)	Dr. Tu-chen Cheng US Army ECBC APG, MD	500-700
	Dendritic polymers	Drs. Ray Yin & Tu-chen Cheng US Army Army Research Lab. & US Army ECBC APG, MD	1700-2000
	Cross-linked enzyme crystals (CLECs)	Dr. Alexey Margolin Altus Biologics, Inc. Cambridge, MA	150-200
	Polyurethane Foam	Dr. Alan Russell University Of Pittsburgh Pittsburgh, PA	100-150
Personnel Protection	Prophylactic use	Drs. Ilona Petrikovoics & James Way Texax A & M College Station, TX	Enhance antidotal protection**
Detection	Biosensor	Drs. Alexsandria Simonian & James Wild TexaxA & M College Station, TX	on going

*Use DFP as substrate; ** see this proceeding.

74. BUFORIN I, A NATURAL PEPTIDE, INHIBITS BOTULINUM TOXIN B ACTIVITY

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The Botulinum (Bot) toxins are composed of seven distinct serotypes (A-G) and are among the most potent toxins to mankind. We have studied Botulinum toxin B (Bot B) serotype, which specifically cleaves between the amino acids GLN (Q) and PHE (F) in position 76-77 of synaptobrevin (VAMP2). We evaluated peptides that contain the QF cleavage site but are not identical in primary structure to the VAMP2 sequence as potential inhibitors of Bot B. Substance P, an 11 amino acid peptide containing the QF bond, was neither a substrate or inhibitor of Bot B in our assay, suggesting that more than the QF bond alone is required. Buforin I (B-I), which contains 39 amino acids and a single QF site (#24-25), did not act as a substrate for Bot B. However, B-I competitively and dose-dependently inhibited Bot B activity, yielding an IC50 of 1 μM. In contrast, Buforin II (a subset peptide of B-I consisting of only 21 amino acids, #16-36) was not a substrate or inhibitor of Bot B activity. We tested two B-I deletion peptides: Peptide36 (36mer; containing B-I amino acids #1-36) and Peptide24 (24mer; B-I amino acids #16-39). Peptide24 exhibited about 25% of B-I activity, while the Peptide36 was nearly 50% as effective as B-I. Sequence analysis of B-I and VAMP2 indicated a similarity of 18% for conserved amino acids. When we compared computer-aided predictions of secondary structure of VAMP2 with B-I, two stretches of helical structure flanking the cleavage site (QF) were found for both sequences. We are now designing and evaluating additional B-I analogs for potent inhibition of Bot B activity. In conclusion, this is the first demonstration of a natural product exhibiting potent inhibition of Bot B.

Keywords: botulinum, toxins, buforin

75.

SCAVENGER PROTECTION AGAINST ORGANOPHOSPHORUS CHEMICAL WARFARE AGENTS BY CHOLINESTERASES

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INTRODUCTION

Current antidotal regimens for organophosphorus (OP) compounds poisoning consist of a combination of pretreatment with spontaneously reactivating acetylcholinesterase (AChE) inhibitor, such as pyridostigmine bromide, to protect AChE from irreversible inhibition by OP compounds, and post exposure therapy with anticholinergic drugs such as atropine sulfate, to counteract the effects of excess acetylcholine, and oximes such as 2-PAM chloride to reactivate OP-inhibited AChE.[1] Although these antidotal regimens are highly effective in preventing lethality of animals from OP poisoning, they do not prevent the post-exposure incapacitation, convulsions, performance deficits, or in many cases permanent brain damage.[2-4] These symptoms are commonly observed in experimental animals and are likely to occur in humans. An anticonvulsant drug, diazepam, was included as a treatment to minimize convulsions, thereby minimizing the risk of permanent brain damage[4]. The problems intrinsic to these antidotes stimulated attempts to develop a single protective drug devoid of pharmacological effects, which would provide protection against the lethality of OPs and prevent post exposure incapacitation.[4]

One approach to prevent lethality and minimize side effects or performance decrements is through the use of enzymes such as cholinesterases (ChEs) as single pretreatment drugs to sequester highly toxic OPs before they reach their physiological targets.[5-14] This approach turns the irreversible nature of the OP-ChE interaction from disadvantage to advantage; instead of focusing on the OP as an anti-ChE, one can focus on the ChE as an anti-OP. Using this approach, it was shown that administration of fetal bovine serum (FBS) AChE or human serum butyrylcholinesterase (Hu BChE) or equine serum BChE protected animals from multiple LD_{50}'s of a variety of highly toxic OPs without any toxic effects or performance decrements.[5-14]

Recently, enzymes been employed as scavengers or prophylactic drugs for protection from highly toxic substances or as detoxifying or decontamination agents. Both, the enzymes for which the toxic agents are substrates that are catalytically hydrolyzed ((e.g., organophosphate hydrolases (OPH) or organophophorous acid anhydride hydrolases (OPAA)), and the enzymes which have a very high affinity for these toxic agents and are irreversibly

inhibited (e.g., ChE) are potential scavengers for OP compounds. There are certain requirements for an enzyme to be an effective scavenger for OP toxicity *in vivo*. It should have a relatively high turnover number, a long half-life *in vivo*, be readily available in sufficient quantities, and not be immuno-reactive. In addition, for enzymes such as ChEs and CaEs, the *in vivo* stoichiometry of sequestration of toxic OP agents should approach 1:1.

The contents of this article describe the progress made in the last decade, by several groups of investigators, in exploring the potential use of enzymes to counteract the toxicity of OPs. Among the enzymes which hold promise as scavengers of highly toxic OP nerve agents, significant advances have been made using ChEs. Since the biochemical mechanism underlying the prophylaxis by exogenous ChEs is established and tested in several species, including non-human primates, this concept should enable a reliable extrapolation of results from animal experiments to human application.

STABILITY OF CHOLINESTERASES *IN VIVO*

ChEs purified from animals such as FBS AChE, equine serum BChE (Eq BChE), and Hu BChE were selected as appropiate forms of bioscavengers to be tested as pretreatment drugs for OP toxicity. Their selection was based on the fact that all three enzymes are soluble globular forms[15,16], easily purified in large quantities from serum[17,18], and have a relatively long half life *in vivo*[6,19-20]. The determination of half-life of all these ChEs in mice[6,20-22], rats[17,22], guinea pigs[14], and rhesus monkeys[13], showed that their mean residence time in circulation was between 35-60 h. The route of administration (iv, ip, or im) affected the time at which the maximum concentration of enzyme in circulation was reached, but did not affect the mean residence time, and a constant level of enzyme was maintained for a period of approximately 3-10 h. Also, regardless of the route of administration, 60-90 % of administered enzyme was found in the circulation of animals.

All recombinant as well as monomeric forms of native esterases tested so far have a relatively low mean residence time in the circulation of mice[20-21]. Therefore, in their present form they are not suitable as scavengers of OPs. In general, only the tetrameric forms of plasma derived ChEs appear to have relatively long residence times in animals. Enzymes isolated from animal species or from plant or bacterial sources may not be suitable for use in humans, for they will cause adverse immune reaction. At the present time, Hu BChE appears to be the most suitable bioscavenger enzyme for human use. Notably, the stability of exogenously administered Hu BChE was determined in individuals identified as being homozygous "silent" for serum BChE and half lives of 8-12 days were reported.[23-25]

SCAVENGER PROTECTION IN RODENTS

The first successful use of AChE or BChE as pretreatment drugs against OP toxicity was demonstrated in rodents[5-8]. For example, pretreatment of mice with FBS AChE[5-8] or Hu BChE[7,22]successfully protected animals against 2-5 X LD_{50} of VX (ethoxymethyl-S-[2-(diisopropylamino)ethylthiophosphonate), or MEPQ (7-(ethoxymethylphosphinyloxy)-1-methylquinolinium iodide), or soman (pinacoloxymethyl-fluorophosphonate) without requiring

any other drug treatment. These studies established a quantitative correlation between the degree of protection against OP compounds and the level of inhibition of administered enzyme. although the protected mice were not evaluated for potential behavioral incapacitation or for any detrimental immunologic response from administering an exogenous enzyme. In addition. these results demonstrated that *in vivo* inhibition of exogenously administered AChE or BChE in blood was proportional to the amount of OP administered as challenge, a result consistent with *in vitro* experiments.

Subsequent studies addressed the question whether pretreatment with a ChE can prevent OP-induced cognitive impairments. Behavioral testing was carried out in rats using the Morris Water Maze Task, evaluating learning, memory, and reversal learning processes. Cognitive functioning in rats significantly impaired following iv administration of 0.9-1.1 X LD_{50} of soman. HuBChE significantly prevented the development of soman- induced cognitive decrements[26]. These results are consistent with previous conclusions that cognitive functions are sensitive to cholinergic manipulations[27,28], Hu BChE treatment alone was devoid of any impairments in behavioral performance, either motor or cognitive. In that respect, it seems that HuBChE has no undesirable performance decrements. These results further support the concept that pretreatment alone with scavenger such as HuBChE is sufficient to increase not only survival but also to alleviate deficits in cognitive functioning after exposure to a potent nerve agent such as soman.

PROPHYLAXIS AGAINST SOMAN INHALATION TOXICITY IN GUINEA PIGS WITH Hu BChE

The use of a ChE scavenger as a prophylactic measure against inhalation toxicity, which is a more realistic simulation of exposure to volatile OPs, has been described by Allon et.al[14]. Hu BChE-treated guinea pigs were exposed to a controlled concentration of soman vapors ranging from 417 to 430 µg/liter, for 45 to 70 seconds. The correlation between the inhibition of circulating Hu BChE and the dose of soman administered by sequential iv injections and by respiratory exposure indicated that ~29% of the inhaled dose of soman reached the blood. A Hu BChE to soman molar ratio of 0.11 was sufficient to prevent the manifestation of toxic signs following exposure to 2.17 X LD_{50} of soman (1 LD_{50} inhaled dose = 101 µg/kg). It was noted that protection was far superior to the currently used traditional approach (pyridostigmine and post exposure therapy). The greater than the calculated values of protection observed was explained by the fact that unlike an iv bolus injection, inhalation exposure allows soman to enter the circulation gradually, which increases the efficacy of soman sequestration to below its toxic levels. The following three important observations are advanced regarding the use of scavengers for OP toxicity[17]: (1) the stoichiometry of protection against inhalation exposure agrees reasonably well with that seen after iv challenge, (2) consistent protection is observed across four species of animals, and (3) the pharmacokinetic behavior of Hu BChE is similar in mice. rats, guinea pigs, and non-human primates.

COMPARISON OF ANTIDOTE PROTECTION AGAINST SOMAN BY PYRIDOSTIGMINE, HI-6 AND ACETYLCHOLINESTERASE

Carbamate, oxime, and enzyme scavenger approaches to protection against highly toxic soman were compared by using the prominent example of each type of antidote[29]. Pyridostigmine in combination with atropine, HI-6 in combination with atropine, and FBS AChE alone were used as examples of carbamate, oxime, and enzyme scavenger antidotes, respectively. Each antidotal regimen produced approximately equal maximal protection against the lethal effects of 952 to 1169 nmol/kg (8-10 X LD_{50}) of soman in mice whose carboxylesterase had been inhibited with CBDP (2-(o-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide). FBS AChE was much better than either pyridostigmine/atropine or HI-6/atropine in reducing post-exposure incapacitation from soman as measured by lacrimation, motor dysfunction, activity level and the inverted screen test. A lower dose of pyridostigmine (566 nmol/kg) or FBS AChE (1150 nmol/kg) was sufficient to protect against 968 nmol/kg (8 X LD_{50}) of soman than was required for HI-6 (200,000 nmol/kg). The circulatory half-life of FBS AChE (1550 min) was much greater than that of pyridostigmine (48 min) or HI-6 (11 min). These results suggest that FBS AChE should be considered a superior alternative to either pyridostigmine/atropine or HI-6/atropine antidotal regimens. The major advantages of bioscavengers for protection against OP toxicity are their rapid removal of OP compounds from circulation and the absence of postexposure incapation and toxic effects that are commonly obesrved in animals protected by traditional antidotal approaches[29].

EXPERIMENTS WITH NON-HUMAN PRIMATES

The successful demonstration of asymptomatic protection of rodents against a variety of OPs by pretreatment with three different ChEs prompted the evaluation of sequestration of OPs by ChEs in non-human primates. The effectiveness of FBS AChE, Eq BChE, and Hu BChE as pretreatment drugs was evaluated in rhesus monkeys, which are more sensitive to OP compounds than rodents. Monkeys were exposed to sarin, or VX, or soman, the OP compound considered to be the most refractory to current therapy[4].

Behavioral performance was measured by a highly sensitive test of cognitive function, the serial probe recognition (SPR) task[9,10,12,30-34]. This behavioral task was chosen because (a) it is a multiple-item memory task that measures short-term memory capacity and decision-making ability[33], (b) it has been used extensively to understand human cognitive processing[34], and (c) it is sensitive to CNS damage in both human and non-human primates[34-35]. For example, rhesus monkeys with damage to the limbic system and humans suffering from amnesia resulting from either Parkinson's or Alzheimer's disease show impaired performance on SPR tasks[34] This task was also shown to be sensitive to disruption after exposure to doses of soman as low as 1.5-2.0 μg/kg[35]. Following iv administration of FBS AChE, the *in vivo* blood AChE activity was elevated more than 100- to150-fold after two hours yet this treatment had very little effect on the SPR performance. The *in vivo* neutralization of soman by FBS AChE (Figure 1) showed a linear relationship between the progressive inhibition of blood AChE activity and the cumulative dose of soman administered[12]. The percent correct and response latencies of monkeys trained on an SPR task to a list length of six items showed complete protection against behavioral incapacitation by soman and no sign of OP toxicity was apparent. There were only 2 out of 4200 trials in which the monkeys failed to respond within the 10-sec interval.

This investigation demonstrated that monkeys displayed minimal adverse reactions from FBS AChE pretreatment. Following OP exposure, even the best pretreatment/therapy regimen. i.e., pyridostigmine pretreatment and atropine/oxime therapy, does not prevent signs of OP intoxication, such as periods of unconsciousness, respiratory distress tremors, and intermittent convulsions.[3-4] The administration of FBS AChE prevented the occurrence of all of these signs of OP intoxication. Thus, the ability of FBS AChE to protect against behavioral incapacitation that results from OP exposure in non-human primates suggests that humans would also be protected.

Concurrently, Broomfield et al.,[9] showed in rhesus monkeys that the toxicity of soman (2 XLD_{50}) can be neutralized by administration of appropriate amount of Eq BChE without any performance decrement as measured by SPR. Also, protection of monkeys against 3-4 X LD_{50} of soman was obtained with Eq BChE pretreatment followed by atropine post exposure treatment. These animals were able to perform the SPR task about nine hour post exposure whereas animals treated with conventional atropine/oxime therapy were not able to perform the same task for 14 days. Animals receiving enzyme alone showed only a subtle transient performance decrement on the SPR task.

A second parameter, the Primate Equilibrium Platform (PEP) task[36-38] was employed to demonstrate the protection of rhesus monkeys from the toxicity of as high as 5 X LD_{50} of soman by pretreatment with FBS AChE or EqBChE without the occurrence of performance deficits[11]. The PEP is a continuous compensatory tracking device that measures the ability of a monkey to compensate for unpredictable perturbations in the pitch induced by a filtered random noise signal. Subjects performed the PEP task for 2.5 h on each soman challenge testing day, and results were presented for each 5-min block of testing time. During the 6 weeks of long-term follow-up, PEP tests were conducted for 2 h; ☞ was computed for each 5-min block of time, and the mean of the 24 resulting data points was calculated to yield one performance score for the entire 2 h.

The iv administration of ~0.5 _mol of ChE alone produced a 100-fold increase in blood ChE activity and caused no apparent physiological or neurological effect or deficit, as measured by the PEP task performance. None of the eight monkeys showed any OP toxicity after soman challenges; protection was so complete that there were no fasciculations even at the site of soman injections. Following the first and second soman injections (totaling 25.6 μg/kg, ~ 4 X LD_{50}), the PEP performance of all eight monkeys was completely normal. The four monkeys pretreated with FBS AChE (Figure 2) or Eq BChE (Figure 3) continued this level of performance even after the third soman challenge. However, the two remaining monkeys that had been pretreated with Eq BChE exhibited a significant but minor PEP deficit after the third soman injection; this transient PEP performance deficit was similar to that observed after exposure of unprotected monkeys to low doses of soman (<2.8 μg/kg)[37-38]. Based upon this comparison, the cumulative protective ratio afforded by ChE pretreatment against soman can be estimated at 10 to 15. During six weeks of post-soman testing, none of the monkeys showed any signs of delayed toxicity, convulsions, or other OP symptoms, or any abnormality on PEP performance[11]. In non-human primates, the 1:1 stoichiometry between ChE and OP dose, plus the endogenous scavenger (ChE, CaE, and other 3-4 unidentified Proteins) present[39-42], the LD_{50} of soman may be extrapolated to be approximately 4.3 ug/kg.

The ability of Hu BChE to prevent toxicity induced by soman and VX was assessed in rhesus monkeys[13]. A molar ratio of Hu BChE:OP ~1.2 was sufficient to protect monkeys against an iv bolus injection of 2 X LD_{50} of VX, while a ratio of 0.62 was sufficient to protect monkeys against an iv dose of 3.3 X LD_{50} of soman, with no additional post exposure therapy. A remarkable protection was also seen against soman-induced behavioral deficits detected in the performance of a spatial discrimination task (Figure 4).

These studies firmly establish that prophylactially administered ChEs, with no additional therapy, prevent the toxicity induced by highly toxic OP nerve agents in mice, rats.

guinea pigs, and rhesus monkeys. Not only do these bioscavengers prevent lethality, but animals do not show any untoward side effects or performance decrements/deficits determined by Morris Water Maze task, SPR task, PEP task, or spatial discrimination task.

Of the three ChEs investigated so far only Hu BChE appears to be an appropriate candidate for exploration for human use. FBS AChE and EqBChE are known to induce the production of antibody when administered in heterologous species of animals (unpublished results). The antibody generated by repeated administration of these two enzymes rapidly clears the circulating exogenous ChEs from blood indicating that the use of such enzymes in heterologous species may not be of much value. The absence of immunological and physiological side effects following blood and/or plasma transfusions in humans and lack of adverse reaction to partially purified Hu BChE administered daily for many weeks[44], support the contention that Hu BChE is the most promising prophylactic antidote. Also, the stability of the exogenously administered Hu BChE in humans (half-life of 8 to 12 days[26-28]) suggests a long-lasting therapeutic level even after administration of a single dose of enzyme.

The systematic evaluation of the efficacy of Hu BChE in protection of four species against nerve nerve agent toxicity[6,7,13,14,26] offered an extrapolation model from animal to human[43]. Based on the stoichiometry of OP sequestration, pre-treatment with Hu BChE predicted protection levels in mice, rats, guinea pigs and monkeys. Further, results show that the stoichiometry of OP sequestration in any given species should depend on the concentration of the circulating enzyme at the time of exposure to challenge. Calculations of protective ratios in humans required quantitative information on the toxicity of OPs in humans. These figures were compiled from the literature describing human volunteer studies with non-lethal doses and accidental exposures to nerve agents, that enabled to obtain an estimation of sign-free doses as well as toxic doses in humans. Predictions were then made by calculating the amount of Hu BChE required to reduce toxic levels of OPs to below the sign-free doses obtained by non-lethal doses, within one blood circulation time in human (seconds).

It was predicted that 200 mg/70 kg HuBChE would protect against up to 2 X LD_{50} of VX or soman, without the need for immediate post exposure treatment[43]. Lowering the dose to 50 mg/70kg is likely to confer protection against long-term exposure to low levels of nerve agents such as soman. It should be noted, however, that the extrapolation from animal-to-human was based on data generated in animals weighing 20 g to 10 kg, and further validation in larger animals may be useful.

IMPROVING THE BIOSCAVENGING CAPABILITY OF CHOLINESTERASES

Many approaches have been made to improve the efficacy of stoichiometric bioscavengers. Enzymes that can hydrolyze OPs are also being considered as promising bioscavengers. These efforts are summarized below:

(a) Amplification of the effectiveness of ChEs for detoxification of OPs by oximes.

A major limitation for use of ChEs as pretreatment drugs for OP toxicity is their 1:1 stoichiometry with OP. An approximately 200-fold difference in molecular weight between

OPs and ChEs, necessitates the use of large amounts of enzyme to provide protection. To improve the efficacy of ChEs as pretreatment drugs, an approach was developed in which the catalytic activity of OP-inhibited AChE was rapidly and continuously restored, by having sufficient amounts of appropriate oxime present[45]. In general, OP-inhibited ChEs can be reactivated rapidly by mono- or bis-quaternary oximes such as 2-PAM and HI-6 so long as it has not undergone aging. The rate of reactivation of OP-inhibited ChE depends on the type and source of ChE, the structure of OP and oxime, as well as the concentration of oxime used. *In vitro* effectiveness of several oximes in reactivating AChE that has been inhibited by a variety of OPs showed that oximes such as, TMB_4, 2-PAM, MMB_4, and HI-6, reactivated AChE inhibited by all OPs to some extent, but HI-6 was the most effective in reactivating AChE that was inhibited by soman and sarin. The capacity of AChE in combination with 2mM HI-6 to detoxify large amounts of sarin *in vitro* is shown in Figure 5a. One mole of enzyme could detoxify a 3200-fold molar excess of sarin or a 64-fold molar excess of soman in the presence of 2 mM HI-6, as compared to a two-fold excess of sarin or soman in the absence of HI-6. Improved detoxification of OP compounds by AChE in combination with oxime has also been demonstrated *in vivo* (Figure 5b). Mice receiving 9 nmol of AChE and 1 mg HI-6 could detoxify a cumulative 57-fold excess of sarin when it was administered by repeated injections at 15 min intervals and so long as the HI-6 level was maintained by repeated injections of 1 mg HI-6[45]. If the level of HI-6 was not maintained, detoxification was less effective as demonstrated by a pronounced decrease in *in vivo* AChE activity.

(b) Site specific mutagenesis of AChE

Several recent studies have demonstrated that it is indeed possible to improve the bioscavenging performance of cholinesterases by site-directed mutagenesis[46]. Using this technique, it is possible to obtain mutant enzymes which possess an increased affinity for OPs,[47] or are more easily reactivated by oximes,[48] and/or possess a reduced rate of aging[46,49-51]. The kinetics of aging was examined in a soman-inhibited mutant enzyme in which the glutamate E202(*199*), located next to the active-site serine S203(*200*) of AChE, was converted to glutamine[46]. For wild-type enzyme, the soman-AChE conjugate aged immediately, giving rise to a form of enzyme resistant to reactivation by oximes. In contrast, the E202(*199*)Q mutant enzyme was largely resistant to aging and could be reactivated by oximes[46-49]. *In vitro* detoxification of soman and sarin by mouse wild-type and E202Q AChE in the presence of 2 mM HI-6 showed that, E202Q AChE was 2-3 times more effective in detoxifying soman and sarin compared to wild-type AchE[46]. These studies show that these recombinant DNA-derived AChEs can offer a great improvement over wild-type AChE as bioscavengers.

To evaluate the possible use of recombinant ChEs as bioscavengers *in vivo*, the mean residence time of five tissue-derived and two rChEs iv injected in mice were compared with their oligosaccharide profiles[20,21] Monosaccharide composition analysis revealed differences in the total carbohydrate, galactose, and sialic acid contents. The molar ratio of sialic acid to galactose residues on tetrameric Hu BChE, rMo AChE, and rHu BChE was found to be ~1.0, suggesting that all the terminal galactose residues were capped with sialic acid. However the mean residence time of Hu BChE was 9- and 14-fold greater than that of rMo AChE and rHu

BChE, suggesting that the capping of galactose with sialic acid by itself is not sufficient to confer circulatory stability to ChEs. For *Torpedo* AChE (mean residence time = 44 min) and monomeric FBS AChE (mean residence time = 304 min), this ratio was ~0.5, suggesting that only half of the terminal galactose residues were capped with sialic acid, yet these enzymes greatly differed in their circulatory stability. In contrast, a molar ratio of 0.5 for sialic acid-to-galactose was observed for the highly stable tetrameric FBS AChE and EqBChE. These observations suggest that although the presence of sialic acid appears to be essential for maintaining ChEs in circulation, the location rather than the number of the non-sialylated galactose residues may be affecting circulatory stability.

Differences in oligosaccharides of ChEs from various sources and the microheterogeneity in glycans on each ChE were elucidated by charge- and size-based separation analyses. However, neither the carbohydrate composition nor the oligosaccharide profile could be completely correlated with the pharmacokinetic parameters of these enzymes. The glycans of recombinant ChEs and monomeric FBS AChE displayed a remarkable heterogeneity in size and consist of hybrid and complex bi-, tri- and tetra-antennary structures. *Torpedo* AChE also contains high-mannose structures. The three plasma ChEs, on the other hand, contain mature glycans which are predominantly of the complex biantennary type, suggesting that these structures are responsible for the extended mean residence times of the enzymes. *Torpedo* AChE, rChEs and monomeric FBS AChE showed a distinctive shorter mean residence time (44-304 min) compared with tetrameric forms of plasma ChEs (1902-3206 min). Differences in the pharmacokinetic parameters of ChEs appear to be due to the combined effect of the molecular weight, and charge- and size-based heterogeneity in glycans. Site-specific analysis of glycan structures may elucidate the structures responsible for the rapid clearance of non-plasma ChEs and suggest suitable manipulations for improving the circulatory stability of rChEs

SUMMARY

The difficulty in providing protection against rapidly aging organophosphorus (OP) agents with current drug regimens led to the development of scavenger protection against OP agents in which a protein scavenger circulating in the blood stream inactivates the OP agents before it can produce its toxic effect. The most extensively tested and developed of these OP scavengers are cholinesterases whose effectiveness in protecting against OP agents has been demonstrated in a variety of rodent and nonhuman primate models. In comparision to atropine/oxime treatment or pyridostigmine pretreatment, fetal bovine serum (FBS) AChE pretreatment provided better protection against postexposure incapacitation in mice challenged with 8 x LD_{50} of soman. Similarly, rhesus monkeys pretreated with FBS AChE, equine BChE or human serum BChE were protected against 3.0-5.0 x LD_{50} of soman and were free of behavioral incapacitation as measured by either serial probe recognition or by a primate equilibrium platform or by spatial descrimination tasks. The effectiveness of scavenger protection in preventing postexposure incapacitation has also stimulated efforts to amplify the effectiveness of enzyme scavenger protection by oxime reactivation of OP-inhibited scavenger. The combination of HI-6 with AChE pretreatment increased the amount of sarin that could be toxified. In addition, combining OP hydrolase with oxime/cholinesterase detoxification extends its efficacy to all nerve agents

and pesticides. The success of recent efforts to increase OP/scavenger stoichiometry by developing AChE mutants that are more resistant to aging or more easily reactivated that wild-type AChE suggests that future OP scavengers will produce even greater levels of medical protection.

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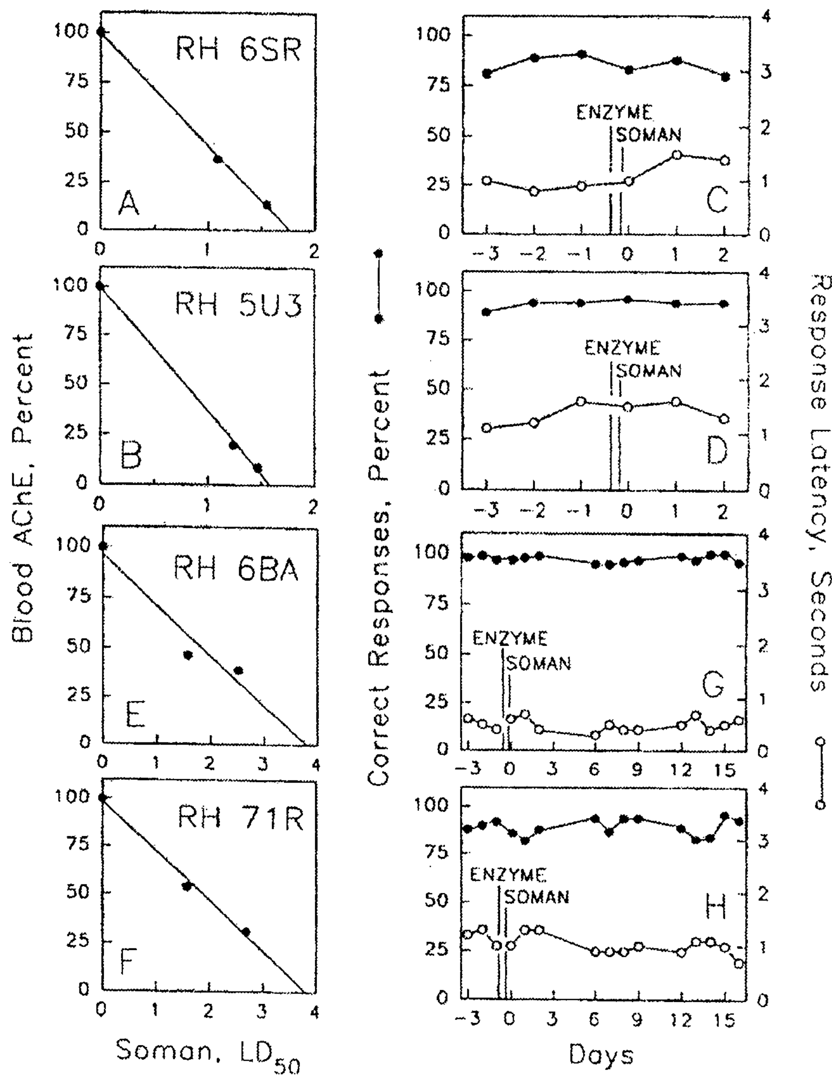


Figure 1. *In vivo* titration of blood AChE in rhesus monkeys pretreated with 105 nmoles of FBS AChE (ABCD). Soman dose shown is the cumulative LD_{50}. Percent correct responses and response latencies for rhesus monkeys. SPR scores (list length of one item) were obtained at indicated times before administration of 105 nmoles of FBS AChE and after challenge with 1.5 LD_{50} of soman, iv, in two injections. *In vivo* of blood AChE in rhesus monkeys pretreated with 210 nmoles of FBS AChE (EFGH). Monkeys were challenged with 2.5-2.7 LD_{50} of soman. Percent correct responses and response latencies for SPR scores (list length of six items) before injection of 210 nmoles of FBS AChE and after challenge with 2.5-2.7 LD_{50} of soman, iv, in two injections. Reproduced with permission from Toxicol. Appl. Pharmacol. (12).

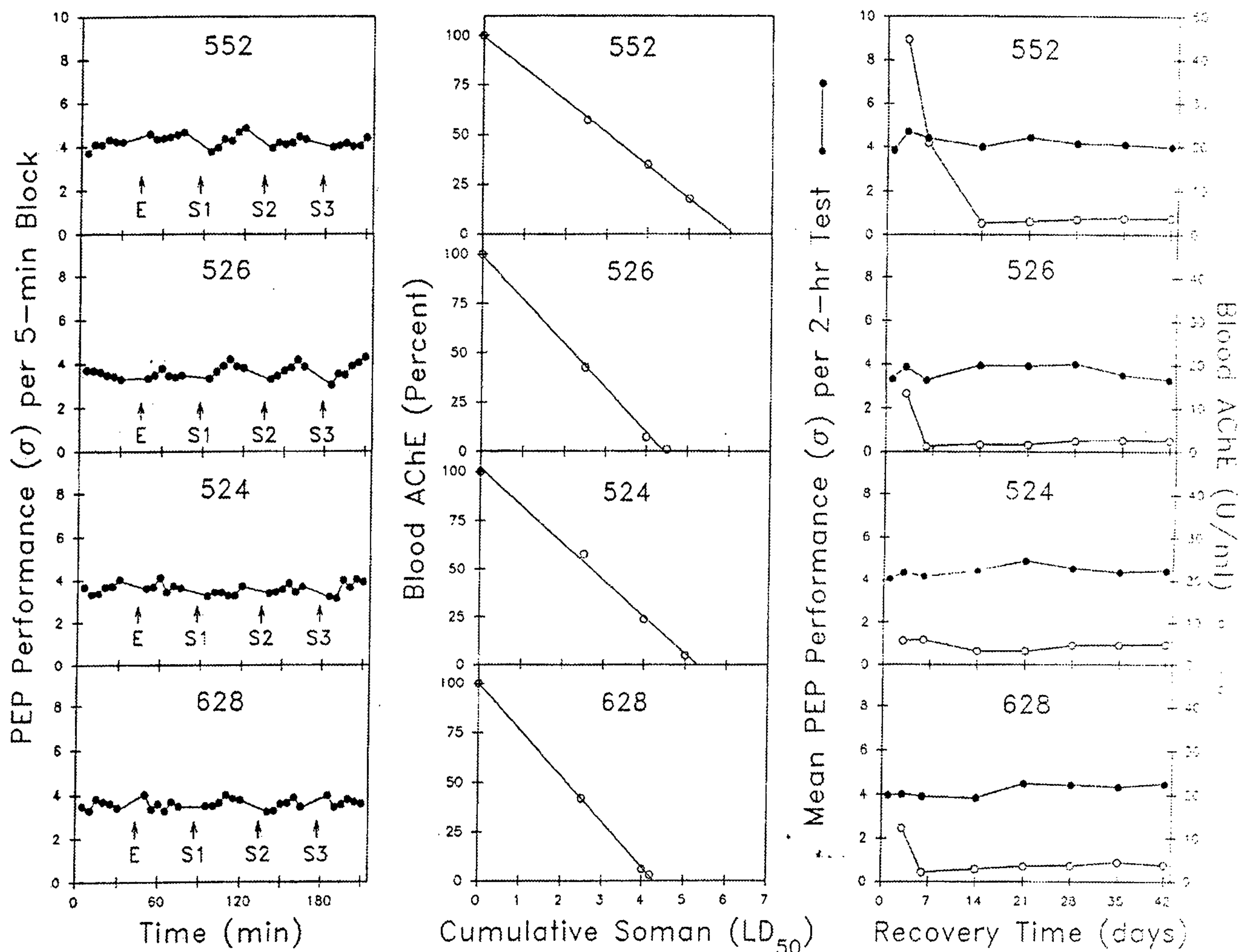


Figure 2 (Left) Effects of i.v. administered purified FBS AChE on PEP task performance before and after challenge with approximately 2.5, 1.5, and 1.0 LD_{50} of soman. Four male rhesus monkeys (5-8 kg), trained to perform the primate equilibrium platform (PEP) task, each received approximately 0.4-0.5 µmol of AChE i.v. (greater than 1:1 stoichiometry with soman). The sequence of behavioral testing and soman challenges was (a) 30-min PEP task (baseline): (b) AChE injection, E (15 min); (c) 30-min PEP task to determine the effect of administration of AChE alone, followed by a 15-min pause for obtaining blood samples, AChE assay, and soman injection, S1 (16.0 µg/kg, ≈2.5 LD_{50}, im); (d) 30-min PEP testing, followed by a 15-min pause for obtaining blood samples, AChE assay, and soman injection, S2 (9.6 µg/kg, ≈ 1.5 LD_{50}, im); (e) 30-min PEP testing, followed by a 15-min pause for obtaining blood samples, AChE assay, and the final im soman injection, S3 (6.4 µg/kg, ≈ 1.0 LD_{50}, was planned but would be reduced if residual AChE activity was judged insufficient); (f) final 30 min of PEP testing. For each 30 min of PEP testing, the data (filled circles) from six sequential 5-min blocks of time are presented. (Middle) *In vivo* titration's of blood AChE in four rhesus monkeys pretreated by iv injection with FBS AChE. Details are as described above. The cumulative dose of soman which reduced ChE activity to the indicated final levels exceeded the amount of AChE administered, suggesting involvement of endogenous esterase. (Right) Long-term effects on PEP task performance of iv administered FBS AChE and challenge with a total of approximately 5 LD_{50} of soman and residual blood AChE levels. PEP performance and blood AChE levels of four monkeys were tested weekly for 6 weeks, filled circles, PEP performance; open circles, enzyme level. PEP performance scores are the mean of data from 24 separate 5-min blocks that compose the 2-hr test. Reproduced with permission from Toxicol. Appl. Pharmacol. (11).

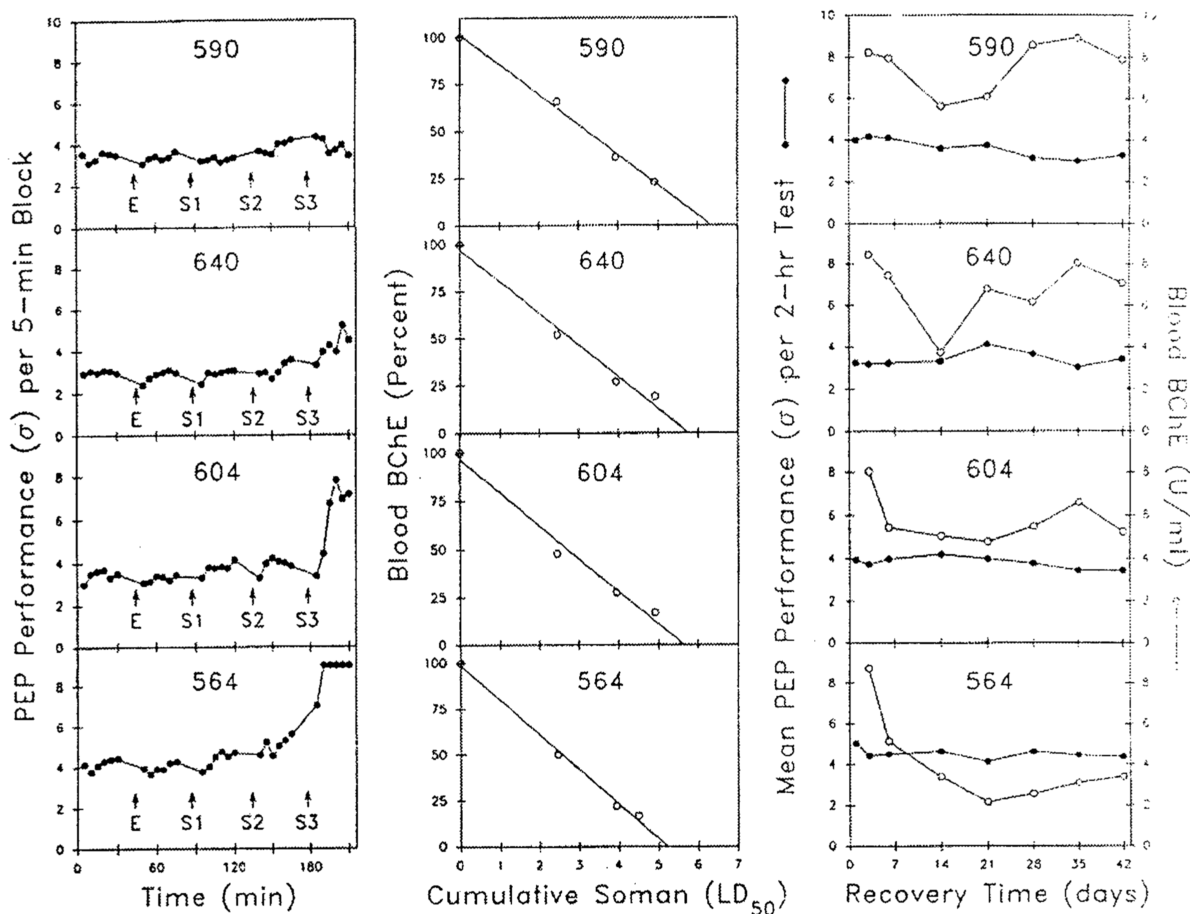


Figure 3. (Left) Effects of iv administered purified horse serum BChE on PEP test performance before and after challenge with approximately 2.5, 1.5, and 1.0 LD_{50} of soman. See legend to Fig. 1. For a detailed explanation. (Middle) *In vivo* titration's of blood BChE in four rhesus monkeys pretreated by iv injection with horse serum BChE. See legend to Fig. 1 for details. (Right) Long-term effects on PEP task performance of iv administered horse serum BChE and challenge with a total of approximately 5 LD_{50} of soman and residual blood BChE levels. Reproduced with permission from Toxicol. Appl. Pharmacol. (11).

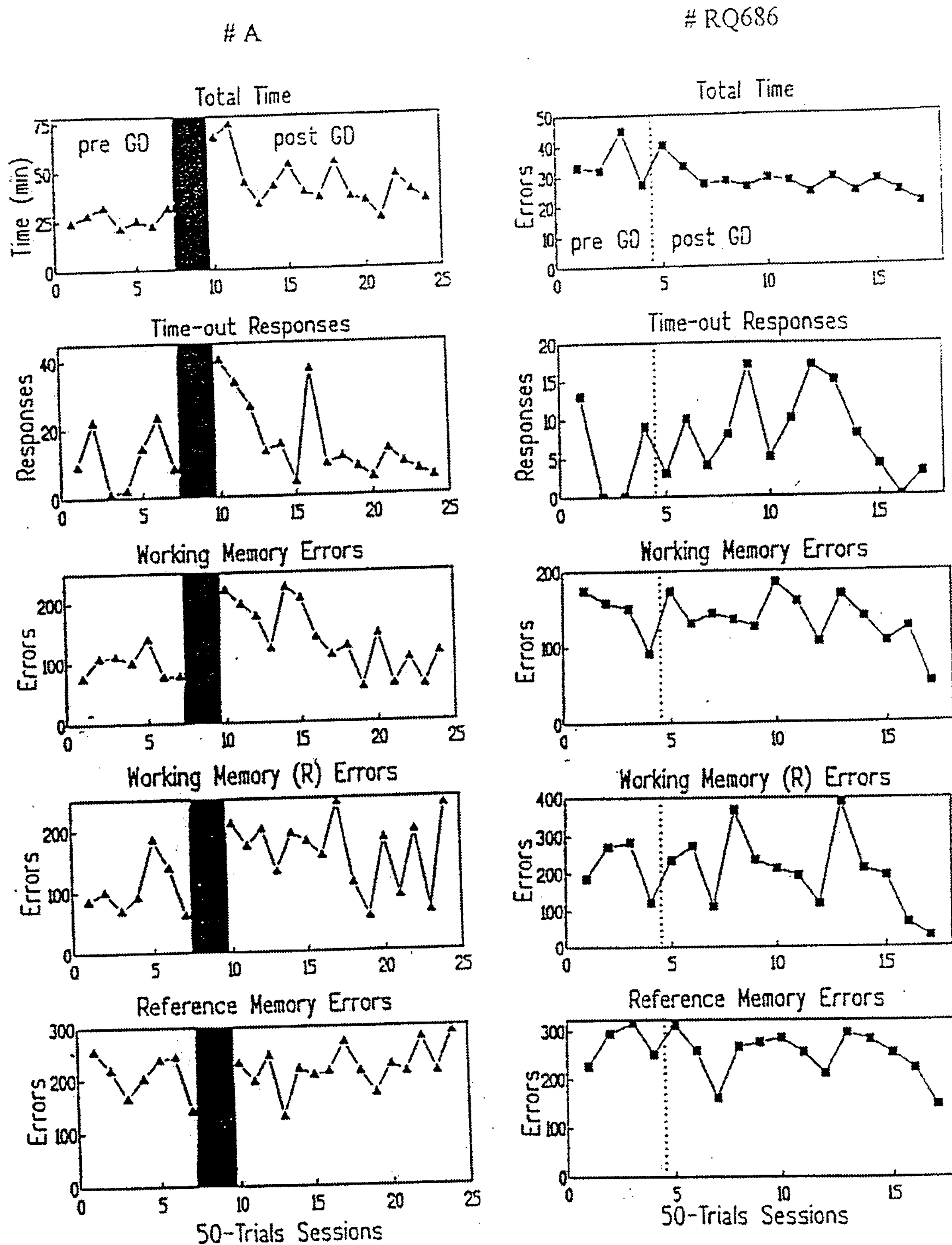
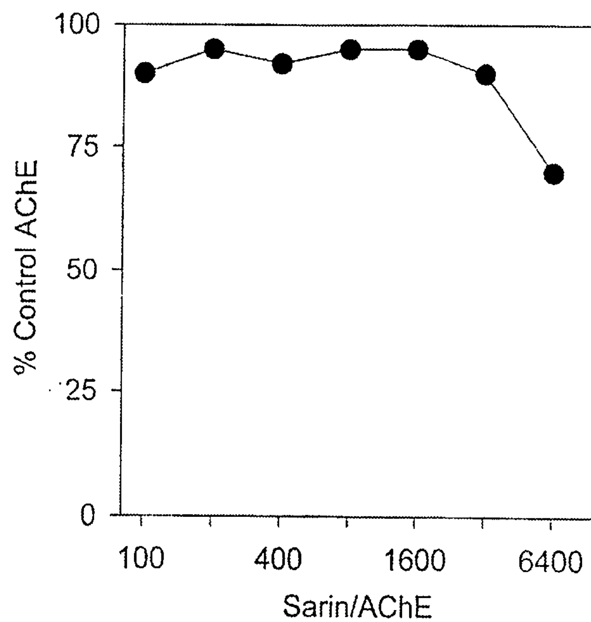


Figure 4. Effects of soman on performance of the spatial discrimination task in monkeys. (Left) Monkey A, pretreated with 0.1 mg/kg pyridostigmine followed with TAB immediately after exposure to 15 µg/kg soman (2.7 x LD_{50}). The shaded area represents 2 days in which no performance was obtained during the presentation of the behavioral tests. The five panels represent the behavioral parameters. (Right) Effects of soman (18 µg/kg; 3.3 x LD_{50}) on performance of the spatial discrimination task in monkey RQ686, following pretreatment with 26 mg HuBChE. No additional treatment was administered. The animal continued its normal performance with no adverse effects immediately following soman (dotted line). Data points are morning sessions only. Note the different scale on the ordinates of the two panels. Reproduced with permission from Toxicol. Appl. Pharmacol. (13).



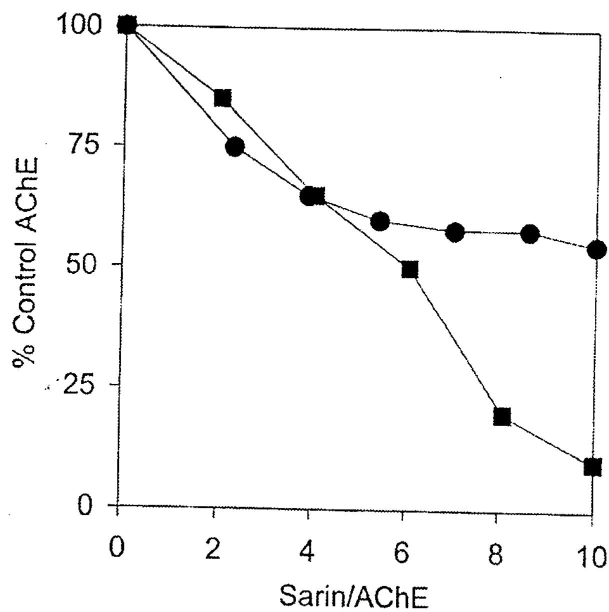


Figure 5a. *In vitro* titration of FBS AChE in the presence of HI-6. Reactivation of FBS AChE (0.125 nmol) in the presence of 2 mM HI-6 at pH 8.0 after repeated additions of sarin at 0.5 hr intervals.

Figure5b. *In vivo* detoxification of sarin by FBS AChE in mice. Mice received IV FBS AChE (9 nmol) followed by sarin (14 nmol) and 1 mg HI-6. Sarin/HI-6 injections (●) or sarin alone injections (■) were then repeated at 15-min intervals. AChE activity was determined 5 min prior to each sarin injection. All mice survived.

76. RAPID, QUANTITATIVE, AND SIMULTANEOUS DETERMINATION OF ACHE AND BCHE LEVELS IN UNPROCESSED WHOLE BLOOD

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ABSTRACT

The concentration of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) in blood is a stable biomarker of suppressed and/or heightened central and peripheral nervous system activity. AChE and/or BChE activity is selectively altered after exposure to nerve agents, organophosphates (OPs), pesticides, anesthetics, terrorists' chemical agents, cocaine, and in some neurodegenerative disease states. Therefore, blood cholinesterase activity can be exploited as a tool for confirming exposure to the agents and diagnosis of diseases.

We developed a robust protocol to quickly and simultaneously provide accurate (greater than 99%) and precise (less than 1%) blood concentrations of both AChE and BChE. This method measures the activity of whole blood in the presence of three AChE and BChE substrates from which the individual AChE and BChE contributions are calculated. This is possible because each protein possesses a different affinity for each of the substrates, and a direct relationship exists between activity and enzyme concentration. To date, we have applied our technique to blood in the presence and absence of selective (huperzine-A and tetraisopropylphophoramide) and non-selective (pyridostigmine bromide) cholinesterase inhibitors. Furthermore, we have used this technique to successfully screen 320 samples in one four-hour time interval.

In conclusion, we developed a method capable of providing fast, precise, and accurate AChE and BChE measurements. Unlike the conventional clinical tests, our procedure provides a more detailed picture of the patient's cholinesterase profile (both AChE and BChE), produces results in less than five minutes, and is capable of screening thousands of samples using state of the art robotics.

INTRODUCTION

Acetylcholinesterase (AChE) is one of nature's most elegantly engineered proteins. The physiological role of the enzyme is the acceleration of hydrolysis of neurotransmitter acetylcholine at nerve-nerve and neuromuscular junctions. The enzyme is ideally suited for this role, because it possesses one of the fastest turnover rates known (1,2). Inhibitors of AChE have been used medicinally in the treatment of glaucoma, myasthenia gravis, and recently, with the FDA approval of tacrine (3) and Aricept (4), Alzheimer's disease. Other inhibitors have been used agriculturally and domestically as pesticides and insecticides. And unfortunately, some of the most potent inhibitors have been developed for chemical warfare. The most recent devastating demonstration of the availability, rapidity and lethality of these agents was the release of sarin into the Japanese commuter train system in Tokyo. Furthermore, since urban terrorism is on the rise, Federal, State and local authorities need a reliable, fast, inexpensive, standard method for confirming such an attack in order to initiate appropriate containment, decontamination and treatment measures.

Current clinical determination of cholinesterase levels within blood includes Michel, microMichel, pH stat, Ellman, and microEllman methodologies (5,6,7). These methods, however, suffer from wide statistical error, long clinical turn around times, lack standardization among labs, and normally determine only the serum or red blood cell cholinesterase concentration. As a result, the reliability of these results is often questioned, and inter-lab comparison is impossible. Worse yet, due to the abnormally long turn around times (up to seven days), physicians are required to treat a patient presenting cholinesterase poisoning long before receiving confirmation. In addition to the clinical methodologies, one field deployable unit is commercially available, the Test-Mate OP system (EQM Research Inc., Cincinnati, OH). Although this unit is fairly user friendly, it possesses several major limitations including numerous processing steps, the use of selective BChE inhibitors, and requires two blood samples and double the processing time for complete AChE and BChE screening.

Therefore, we have developed a new methodology that quickly and simultaneously determines the concentrations of AChE and BChE in unprocessed, whole blood. Unlike the conventional clinical tests or the Test-Mate OP unit, our method provides a full analysis of the patient's cholinesterase levels, does not rely on the addition of selective AChE or BChE inhibitors, uses a single non-invasive blood collection technique (finger prick), is not labor intensive, and produces results in less than five minutes. We have circumvented the aforementioned problems by simultaneously determining the levels of AChE and BChE in aliquots from the same blood sample.

This is possible because blood contains two cholinesterases that possess different affinities for any given substrate, and a linear correlation exists between enzyme activity and concentration. Thus, if one measures the activity of any given blood sample with two different substrates, then it is feasible to calculate the precise concentrations of both proteins (i.e., two equations with two unknowns). Furthermore, monitoring the activity with three substrates (equations 1-3) provides three fold degenerate data [i.e., three sets of two equations (equations 1&2, 2&3, 1&3) with two unknowns]. In equations 1-3, the rates of substrate hydrolysis are represented by R1, R2, or R3 and correspond to the turnover of substrate 1, substrate 2, or substrate 3, respectively. The [AChE] and [BChE] refer to the actual concentrations of AChE and BChE contained in the sample. Finally, the coefficients in each equation (i.e., x1, x2, x3, and y1, y2, y3) represent the sensitivity coefficients. These coefficients represent the contribution that AChE and BChE contribute to the overall rate of substrate hydrolysis (R1, R2, R3).

$$R1 = x1[AChE] + y1[BChE] \quad (1)$$
$$R2 = x2[AChE] + y2[BChE] \quad (2)$$
$$R3 = x3[AChE] + y3[BChE] \quad (3)$$

Simultaneously solving the three sets of degenerate equations provides three independent estimates for the concentrations of AChE and BChE. Therefore, determining the mean value and the standard deviation for the three independently derived values provide an excellent estimate of the authentic concentrations of each protein

MATERIALS AND METHODS

Acetylthiocholine iodide (ATC), propionylthiocholine iodide (PTC), butyrylthiocholine iodide (BTC), 4,4'-dithiopyridine (DTP), and tetraisopropylphosphoramide (Iso-OMPA) were purchased from Sigma Chemical Co (St. Louis, MO). Racemic huperzine-A (Hup-A) was purchased from CalBiochemical-NovaBiochem Corporation (San Diego, CA). Water was polished to 18.2 mega-ohm by passage through a Millipore purification system (Millipore, Bedford, MA). Trunk blood obtained from 10 Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) was stored in the presence of EDTA. All blood samples were refrigerated at 4 C until used.

The following stock reagents and buffer were prepared and stored at -20 C until needed, or stored at 4 C when in use. Thirty mM ATC (sATC), 30 mM PTC (sPTC), and 30 mM BTC (sBTC) were prepared in 18.2 Mega-ohm water, and 6 mM DTP (sDTP) was prepared in 10% HPLC grade methanol/buffer. The buffer consisted of 50 mM sodium phosphate, pH 8.0. Working reagents were prepared in buffer immediately prior to use and stored at room temperature. These reagents were 1.0 mM ATC and 200 DTP (A), 1.0 mM PTC and 200 μM DTP (P), 1.0 mM BTC and 200 μM DTP (B), and 200 μM 4,4'-dithiopyridine (D). Solutions of the selective inhibitors Hup-A (0 to 900 nM), Iso-OMPA (0 to 5.12 μM), and both (0 to 900 nM Hup-A and 0 to 5.12 μM Iso-OMPA) were prepared in water. In addition, serial dilutions of each species whole blood were prepared in water.

All species' blood and dilutions were titrated using a series of inhibitors. 10 μL of an inhibitor solution was transferred to a PCR tube followed by the quantitative addition of 150 μL of a whole blood dilution. The contents were thoroughly mixed and incubated at room temperature for three hours. Then, 10 μL aliquots from each PCR tube were transferred repetitively to one row of a standard 96 well microtiter plate (Corning, Acton, MA). Next, 290 μL aliquots of D (control) were added to columns 1-3, 290 μL aliquots of A were added to columns 4-6, 290 μL aliquots of P were added to columns 7-9, and 290 μL aliquots of B were added to columns 10-12. An assay was performed on the plate using a Molecular Devices SpectraMax Plus microtiter spectrophotometer (Sunnyvale, CA). The first was a four-minute kinetic assay in which the change in absorbance at 324 nm was monitored. Other parameters included sixty second pre-read mixing, three second shaking between reads, twelve second intervals, and linear least squares data analysis. Subsequent cholinesterase screening was performed as described above unless otherwise noted, except that the samples were prepared solely by dilution with water.

RESULTS

A representative titration of guinea pig blood with a selective inhibitor (Hup-A) is depicted in Figure 1A. In this Figure, titrations at several blood dilutions for ATC turnover are displayed. Each titration was fit via nonlinear least squares analysis to a simple binding isotherm possessing a residual velocity. Note that all rates were corrected for blood hydrolysis of DTP. The control velocities, Vc, and the residual rates, Vr, for each substrate (i.e., ATC, PTC, and DTP) were graphed as a function of blood concentration, Figure 1B. The resulting relationships were fit via linear least squares analysis then tabulated in Table 1. The slopes for each Vr relationship corresponds to the contribution that BChE contributes to the corresponding control reaction and are the sensitivity coefficients (SC). The SC for each substrate for AChE is calculated by subtracting the corresponding BChE SC coefficient from the control. The sensitivity coefficients for AChE and BChE for each substrate are contained in Table 2. Similar results

were also obtained for the Iso-OMPA titration, except that the Vr slopes correspond to the AChE SC, and the BChE SC are obtained by subtracting those for AChE from the corresponding Vc relationship. The SC determined from the Iso-OMPA titration, and the average values from both titrations are contained in Table 2. In reference to equations 1-3 above, x1, x2, x3 and y2, y2, y3 correspond to the sensitivity coefficients of AChE and BChE for ATC, PTC, and BTC, respectively. The observed rates for ATC, PTC, and BTC likewise correspond to R1, R2, and R3, respectively.

The observed rates of hydrolysis for the inhibitor solutions consisting of Hup-A, Iso-OMPA, and the combination of Hup-A and Iso-OMPA were converted into U/mL. This was accomplished via equations 1-3 and the average sensitivity coefficients for AChE and BChE (cf. Table 2). The processed AChE and BChE concentrations for the Hup-A and Iso-OMPA titrations are contained in Table 3. The results for the 8x diluted blood sample of guinea pig are plotted in Figure 2. In this Figure, panel A refers to the results from the Hup-A titration, panel B corresponds to the Iso-OMPA titration, panel C corresponds to the AChE results for titration by the combination mixture (Hup-A and Iso-OMPA), and panel D depicts the BChE results for the combination mixture. It is important to note that for ease of comparison with panels A and B the axis in panels C and D have been chosen to reflect the response due to Hup-A and Iso-OMPA, respectively. In other words, the AChE response was plotted as a function of Hup-A rather than Iso-OMPA, and BChE was plotted as a function of Iso-OMPA rather than Hup-A.

DISCUSSION

As clearly demonstrated by Table 2, the sensitivity coefficients for AChE and BChE are identical regardless of which titrant is used. This indicates that all observed activity for ATC, PTC, and BTC hydrolysis is due to the combined actions of AChE and BChE. Thus, use of the sensitivity coefficients as detailed in the background section is feasible. Although it is possible to use only two substrates for analysis, it is preferred that three be used. It is our opinion that the use of three substrates provides redundant and independent determinations of the concentration of both AChE and BChE. The average reflects a statistically better representation of the true value than any single determination. Additionally, the standard deviation associated with mean provides is very informative. First, it provides an internal validation of the assay. If the protocol were flawed, large fluctuating standard deviations would be expected. This does not occur as per Table 3, which possesses a nearly constant standard deviation of 0.003 U/mL. Second, it indicates how precisely the concentration of each protein is known. The average precision of this assay is 0.7% and 0.3% for AChE and BChE, respectively (cf. Table 3). For AChE and BChE, this is the average percent confidence value for the eight separately calculated concentrations of AChE in the Iso-OMPA and Hup-A titration data, respectively. Sample to sample variation (repetitive determinations) is also contained in this data. The precision for AChE is roughly 3% and for BChE the precision is about 1.4% (i.e., the standard deviation of the eight independently determined concentrations of AChE and BChE in the presence and absence of Iso-OMPA and Hup-A, respectively).

Since pure samples of guinea pig blood AChE and BChE were not available, the accuracy of the method was assessed indirectly. Since Hup-A is selective for AChE, the loss in activity with respect to a control reaction at infinite inhibitor concentration corresponds to the concentration of AChE contained in the sample. Likewise, the concentration of BChE in the sample can be extracted from Iso-OMPA titration. The uninhibited data in Table 3 was compared to the calculated theoretical concentrations of AChE (0.610 U/mL) and BChE (0.911 U/mL). The accuracy for AChE and BChE determined using this method is greater than 99% for both AChE and BChE. Although not included in this paper, similar results are obtained for other species over several titrations. Obviously, obtaining pure AChE and BChE for spiking blood samples with known amounts would be more desirable; however, an indication of the accuracy has nonetheless been presented.

Finally, blood samples inhibited by mixtures of Hup-A and Iso-OMPA produced identical results to those obtained independently using pure Hup-A and pure Iso-OMPA solutions. This is clearly demonstrated comparing the data and theoretical curves of AChE titrated by Hup-A alone (2A) to those in the presence of both inhibitors (Figure 2C). In addition to visual inspection, the calculated inhibition constants are 1.6 +/- 0.1 nM and 1.7 +/- 0.1 nM in the absence and the presence of Iso-OMPA, respectively. Comparing panels B (Iso-OMPA alone) and D (Hup-A and Iso-OMPA) in Figure 2 provides similar results for BChE. The calculated IC50 values are 13 +/- 3 nM and 10 +/- 3 nM in the absence and the presence of Hup-A, respectively. Obviously this method is capable of discriminating individual effects on AChE and BChE activities in the presence of selective and non-selective cholinesterase inhibitors.

CONCLUSION

In conclusion, a methodology capable of screening unprocessed whole blood samples has been presented. The unprecedented precision (<1%) and accuracy (>99%) of this procedure is unmatched by current clinical practices, and unlike the current methods, does not suffer from large statistical fluctuations nor long turn around times. Additionally, this protocol provides a much more detailed picture of an individual's cholinesterase profile using non-invasive sampling techniques. Although not discussed in this paper, initial validation with the COBAS/FARA, TestMate OP, and Michel methods is underway, and the preliminary results demonstrate linear correlations between all methods at a high confidence level (an average regression coefficient of about 0.99 for both AChE and BChE). In addition, the sensitivity coefficient method has been applied to several species including human, Rhesus monkey, and Sprague Dawley rats. Finally, the simplicity, precision and accuracy of this protocol will allow clinicians to screen and confirm suspected ChE intoxication with unprecedented confidence, and therefore be able to initiate, follow, and dynamically adjust treatment. It is anticipated that further refinement of this technique will provide a universally accepted, real time, inexpensive method for simultaneously screening whole blood samples for AChE and BChE.

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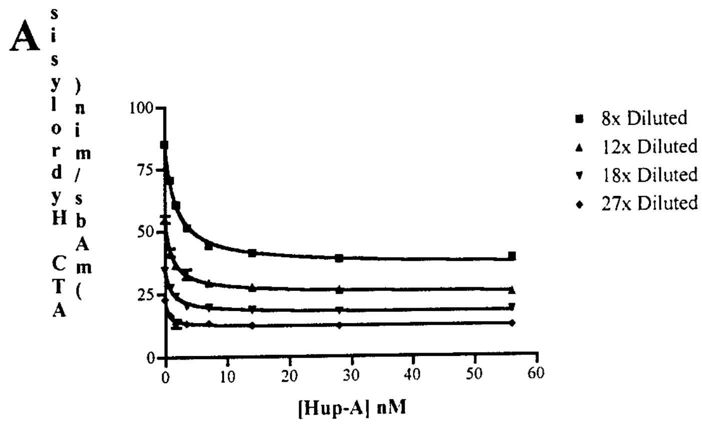
KEYWORDS
Cholinesterase, screening, chemical warfare agents, biomarker, inhibitor

FIGURES AND TABLES



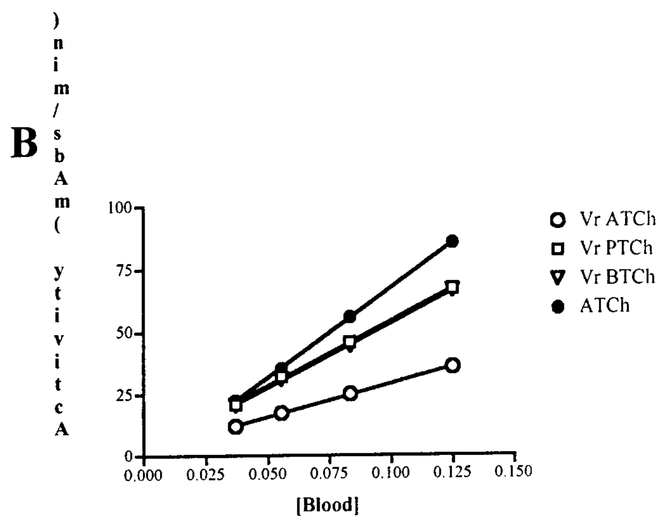


Figure 1. A representative titration of guinea pig blood with a selective inhibitor (Hup-A).

Figure 2. The processed AChE and BChE concentrations for the Hup-A and Iso-OMPA for the 8x diluted blood sample of guinea pig.

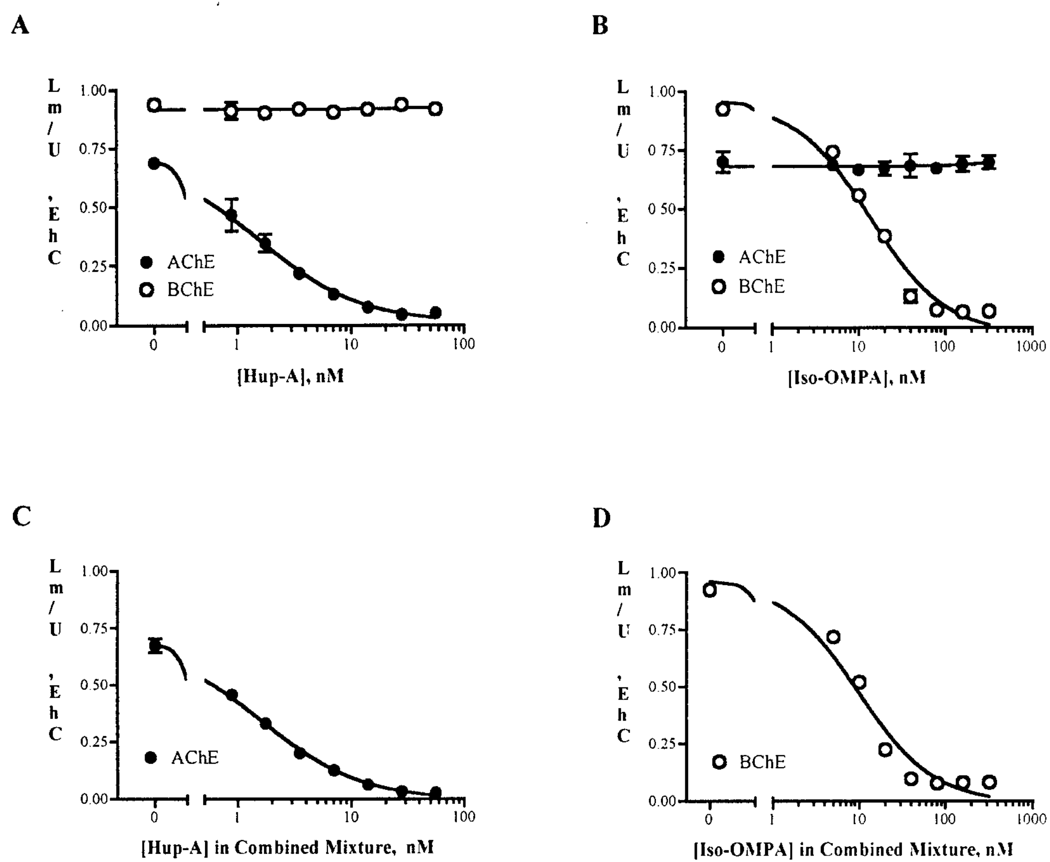


Table 1. Slopes of Control Reactions and Residual Activities

	Control		Hup-A	
	mAbs/min/[Blood]	Error	mAbs/min/[Blood]	Error
ATC Slopes	715	7	270	4
PTC Slopes	744	11	518	12
BTC Slopes	557	10	515	9

Table 2. Hartley Guinea Pig Blood Sensitivity Coefficients

		AChE mAbs/min/[Blood]	BChE mAbs/min/[Blood]
Hup-A Titration	**ATC**	444	270
	PTC	226	518
	BTC	43	515
Iso-OMPA Titration	**ATC**	418	297
	PTC	194	550
	BTC	0	557
Average	**ATC**	431	284
	PTC	210	534
	BTC	21	536

Table 3. Processed Titration Results (Activity to U/mL)

	[AChE]		[BChE]		
[Hup-A], nM	**Value**	**STD**	**Value**	**STD**	**% CV**
56	0.006	0.004	0.907	0.004	0.4
28	0.019	0.002	0.929	0.002	0.2
14	0.055	0.003	0.912	0.001	0.1
7	0.136	0.002	0.892	0.002	0.2
4	0.214	0.002	0.907	0.002	0.2
2	0.334	0.002	0.93	0.003	0.3
1	0.462	0.001	0.913	0.002	0.2
0	0.61	0.007	0.925	0.004	0.4
			1.4		0.3
			%CV		**Average**

	[AChE]			[BChE]	
[Iso-OMPA], nM	**Value**	**STD**	**% CV**	**Value**	**STD**
320	0.605	0.007	1.2	0.048	0.003
160	0.63	0.003	0.5	0.071	0.001
80	0.616	0.001	0.2	0.08	0.001
40	0.635	0.004	0.6	0.143	0.001
20	0.617	0.004	0.6	0.279	0.005
10	0.585	0.004	0.7	0.565	0.003
5	0.597	0.005	0.8	0.699	0.001
0	0.582	0.008	1.4	0.945	0.004
	3.2		0.7		
	%CV		**Average**		

77. NOVEL STRATEGY USING SYNTHETIC NUCLEIC ACIDS AND CONVENTIONAL IMMUNOASSAYS FOR BIOLOGICAL AGENT DETECTION

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ABSTRACT

Detection of biological agents that could be released domestically by terrorists, or during military conflict, poses several challenges. Because of the unknown identity of the potential biological threats, it is necessary to provide simultaneous alert for all known agents. This necessitates the need to perform up to several hundred concurrent assays on a single sample. The assay needs to have multiplicity of several distinct identifying markers for each suspect agent; thereby limiting the number of false positive and false negative results. Moreover, the assay needs to be able to perform this identification in the shortest time possible as initiation of efficacious treatment protocols, as well as limiting spread of agent, is more effectual in limiting the overall human impact. To accomplish this, we propose a synthesis of two areas of biotechnology; one well established, the other now rapidly expanding; antibody immunoassays and DNA "chip"-based analysis, respectively. The theoretical development of a detection system that employs a standard immunoassay, coupled with a particle suspension, which contains (non-infectious) molecular analogs of the targeted biological agents, and finally synthetic DNA segments (specifically designed as a unique agent identifier) attached to the molecular analogs, is the focus of our research. As will be discussed in greater detail, the use of such a system could potentially allow for a capacity to test for multiple biological agents accurately and simultaneously. The use of synthetic DNA tags, as the means to evaluate the results of a simple immunoassay, is the novel conceptual innovation of this proposed strategy.

INTRODUCTION

To date, no technology exists to perform the scale of concurrent tests described above. In order to provide equivalent breadth and accuracy, 5 x 100 conventional assays would be performed on 500 aliquots of a single sample. Assuming sufficient volume of sample is available, this assay strategy would require a minimum of 500 – 1000 man-hours of effort. It is probable that incremental improvements in productivity of conventional assays can be accomplished by combining a limited number of assays in one system. However, the capacity to test for up to 100 threats accurately and simultaneously requires a new approach to assay development. The stated goals of our proposed methodology are challenging. Nevertheless, each of the technical components of the proposed strategy is an established capability; the novelty arises from the manner in which these components are combined.

We have termed the proposed strategy the **M**assively **P**arallel Immuno**a**ssay (**MP*A**). This terminology is derived from the current design of powerful supercomputers that are composed of "massively parallel" arrays of processors that accomplish high cumulative rates of computing production by dividing a single computational job into separate tasks that are assigned concurrently to different processors. An asterisk (*) is employed to indicate that the mature technology will ultimately be Ligand independent; i.e. may concomitantly use several Ligand chemistries.

DISCUSSION:

*Strategy of MP*A*

The proposed project is based on the combination of two areas of biotechnology, one very well established and the other now rapidly expanding. The better-established area is that of antibody biotechnology, which is currently a multi-billion dollar industry, based largely on the application of immunodiagnostics in health care. Such tools currently find extensive clinical applications in evaluating endocrine function, diagnosing bacterial and viral disease, and establishing the safety of transfusion blood units by testing for hepatitis and HIV contamination.

The second biotechnical innovation that enables timely development of the proposed assay system stems from advances in DNA biotechnology, with specific emphasis on "chip"-based analytical technology. Recent years have seen the development of proven miniaturized systems capable of a broad spectrum of analyses ranging from biochemical assays and DNA-based diagnoses to genomic sequence determination. (1,2,3,4) We plan to use this capability, not to analyze DNA changes in living cells, but to use synthetic DNA fragments as a means to sort out the results of hundreds of immunoassays processed simultaneously in a single small sample.

Conventional biomolecular test systems possess limited assay scope. To bypass this limitation we propose a strategy that is based on an unconventional application of a standard immunoassay detection method: i.e., the

competitive or displacement assay. Briefly, the detection system will consist of an ensemble of suspended ferromagnetic micron-scale particles, each of which will display on its surface antibodies with high-affinity recognition properties for a specific molecular site on a particular toxin, virus, or bacterium of interest. The use of ferromagnetic particles allows for magnetic manipulation of the system; for instance, the beads can be held in place while the surrounding liquid is moved to another site. However, other means to separate liquid from solid particles are available; filtration, for instance, may prove to be more reliable if a significant rate of escape from the magnetic trap is experienced.

For each toxin, virus, bacterium, or chemical target to be surveyed, several different antibody-particle units are to be included, each of which recognizes the target via a different site on the molecule. Multiple independent responses to the presence of the target in a test sample provide protection against false positive and false negative results. These (primary) antibodies will have high binding affinity for the target molecule. The particle suspension will also contain soluble (non-infectious) molecular analogs of the targeted biological agent. Particle-based antibodies bind these mimics or proxies with moderate affinity; i.e. antibody-proxy complexes dissociate and reform several times each minute. Although proxies could be a non-toxic variant of the target molecule, in practice we expect routine generation of proxies to be systematically achieved by generation of antibodies that interact with the binding site of the primary antibody. In this way, generation of proxies could become at least semi-automated. To each analog or proxy is attached a relatively short segment of DNA of defined nucleotide-base sequence. Each sequence of DNA is assigned specifically to one particular analog/target pair and serves as a serial number or 'bar-code' in this testing strategy. Attachment of DNA segments to protein is straightforward and easily accomplished by incorporating an activated nucleotide at a position that is not involved in binding to the matrix(5). Each DNA segment has a fluorescent compound for subsequent visualization attached to it. Figure 1

If a test sample contains quantities of one or more of the target molecules, such molecules will be bound by the corresponding set of specific antibodies during intervals in which the antibody is free of bound proxy. Because of the high affinity between antibody and authentic target, the antibody will not be available for further binding of proxies, resulting in the permanent displacement of labeled analogs. Sample is added to the suspension of antibody-linked beads after a short incubation time (to be determined). The beads will then be separated from the surrounding liquid. In a ferrous microparticle-based system, beads would be immobilized by a magnetic field, while the liquid is to the DNA hybridization chip.

Each displaced proxy molecule will accumulate at a specific position on the DNA chip. We assume that the protein proxy will not interfere with DNA interaction with its complementary site on the chip(6). Should there prove to be interference, the DNA-protein linkage will be based on a chemically labile disulfide bond that can be chemically reduced to separate protein from DNA. The DNA on the chip will be visualized by the accumulation of an attached fluorescent label. It is possible that the larger protein component, while not interfering with DNA attachment, could obscure observation of fluorescence, in which case it would also be useful to decouple the DNA fragment from the protein proxy.

Because multiple tests for a single target will be performed simultaneously, the presence of an authentic biological threat will be internally confirmed by the concurrent accumulation of fluorescent signal at several sites. For instance, assume five independent tests for a particular target, such as a bacterial toxin. A perfect result, if the sample contained the toxin, would generate five points of fluorescence on the detector. Fluorescence at four of the five positions would also be interpreted as a positive signal; the failure to observe fluorescence at the fifth position could signal that the toxin is a normal genetic variant that is not reactive with one of the antibodies. Alternatively, the toxin might have been genetically engineered to avoid detection by altering the structure of the molecule's surface. Multiple probes reduce the probability of missing modified toxins. Note that certain parts of the molecule cannot be modified without attenuating its biological effect; a mature test system will include antibodies that recognize functionally critical portions of the toxin or other target. Routinely, if only one or two sites on the DNA detector show development of fluorescence, the inference of presence of toxin in the sample would not be drawn. However, if these sites correspond to functionally critical sites on the molecule, a conclusion of possible presence of the toxin would be reasonable, leading to further testing, perhaps by other means. This use of synthetic DNA tags, as the means to evaluate the results of a simple immunoassay, is the unique innovation of the proposed strategy. Keeping in mind, however, that the effective exploitation of DNA as a biotechnological tool has been demonstrated(7). As short segments of DNA can be designed and synthesized to form stable complexes. The technology to produce DNA chemically and to monitor specific complex formation, or hybridization, is already well established.

SUMMARY

A mature **MP*A** system will be fully automated. It is premature to attempt to describe the final outcome in detail: the purpose of the proposed research was to establish the scientific basis for the assay strategy. However, it is likely that proposed design goals, implemented during commercial production, could include configuring the system such that all necessary reagents for testing up to 100 or more samples would be packaged in a sealed cassette of relatively small size. The cassette would be inserted into a device that would have the mechanical and computer systems to control all manipulations and to analyze resulting data. Samples would be loaded into a cartridge and inserted into the device by the technician. Inside the unit, the cassette will be opened; samples will be drawn from the cartridge and assayed. Liquid transport is envisioned as being accomplished by syringe pumps incorporated within the cassette and manipulated by the assay unit. Each DNA chip can be cleaned and reused multiple times during each run. All liquids will remain within the cassette, probably including the sample cartridge. Upon completion of the assay, the cassette will be relocked and ejected. Although beyond the scope of the proposed project, it is probable that sample preparation itself could be performed in a largely automated manner. From a hypothetical standpoint, for some applications, the entire operation could be performed independently and the results could be reported remotely.

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KEYWORDS
Biological Agents, Immunoassay, Molecular Analogs, Synthetic DNA, DNA tag

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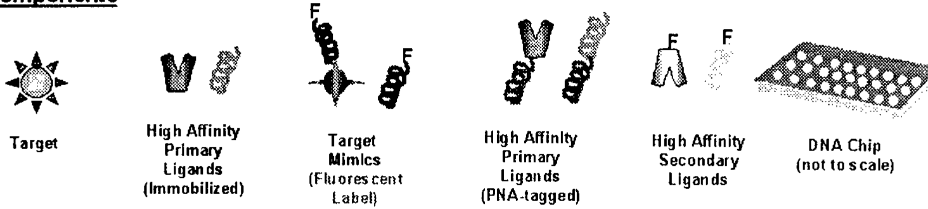
FIGURES AND TABLES

MP*A Basic Assay Strategy

Components



Protocol Combine test sample and assay components
Incubate (5-10 min)
Transfer non-immobilized components to DNA Chip / read out

Post-Assay Configurations

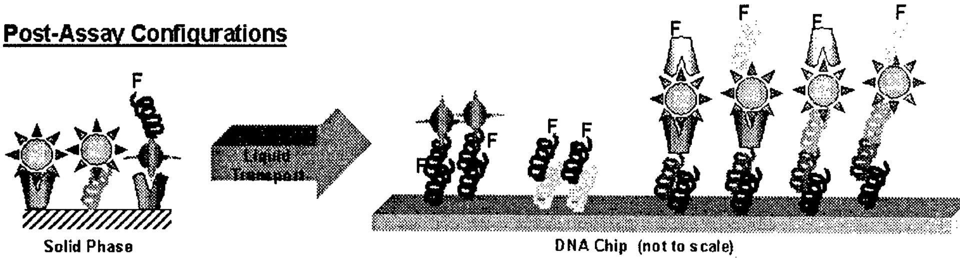


Figure 1: Basic Strategy for Massively Parallel Immunoassay

78. OP NERVE AGENT DECONTAMINATION, DETOXIFICATION, AND DETECTION USING POLYURETHANE IMMOBILIZED ENZYMES

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ABSTRACT
As an extension of the bioscavenger approach to the protection against organophosphate toxicity, we developed a sponge product, composed of polyurethane immobilized ChEs (AChE and BChE) and organophosphate hydrolases, and oxime for decontaminating OPs from sensitive biological surfaces. The ChE-sponge is also a biosensor for OPs so troops can rapidly determine OP exposure and contamination. The enzyme products exhibit remarkable mechanical and chemical stability when immobilized and do not leach from the synthesized matrix, yet retain the function of their soluble counterparts. For example, DFP and MEPQ reacted with the immobilized ChEs, and rinsing the sponge with HI-6 restores cholinesterase activity, permitting the AChE-sponge to be recycled many times. Since OPs need to be wiped onto the sponge to be detoxified, several sponge formulations have been developed to rapidly remove soman from guinea pig skin. Using this enzyme-sponge technology, we are developing a rapid and simple kit to detect OP contamination on humans, in water or almost any environment. ChEs and non-ChE enzymes have been immobilized to yield small OP sensitive and selective biosensors. For long-term OP detection, ChE-biosensors were continuously exposed to untreated natural fresh or salt water over 60 days at room temperature: the badges retained 80% of their original activity. In conclusion, immobilized ChEs retain high activity and increased stability, making them suitable for a variety of detoxification and decontamination schemes for both chemical weapons and pesticides directed against ChEs, and as biosensor badges to immediately detect or monitor long-term OP contamination, for example in drinking water.

INTRODUCTION
It was previously demonstrated that a variety of enzymes exhibited enhanced mechanical and chemical stability when immobilized on a solid support, producing a biocatalyst. The study of degradation of organophosphates by immobilized enzymes dates back to Munnecke (1), who attempted to immobilize a pesticide detoxification extract from bacteria by absorption on glass beads. The absorbed extract retained activity for a full day. Wood and coworkers (2), using isocyanate-based polyurethane foams (Hypol®), found that a number of enzymes unrelated to OP hydrolysis could be covalently bound to this polymer; after that Havens and Rase (3) successfully immobilized parathion hydrolase. More recently, the enzyme bioscavenger approach (4, 5) has been shown to be effective against a variety of OP compounds *in vitro* and *in vivo*; pretreatment of rhesus monkeys with fetal bovine serum (FBS) acetylcholinesterase (AChE) or equine serum butyrylcholinesterase (BChE) protected them against a challenge of up to 5 LD_{50} of soman. While the use of cholinesterase (ChE) as a single pretreatment drug for OP toxicity provided complete protection, a stoichiometric amount of enzyme was required to neutralize the OP *in vivo*. To increase the OP/enzyme stoichiometry, enzyme pretreatment was combined with oximes such as HI-6 so that the catalytic activity of OP-inhibited AChE is rapidly and continuously restored before irreversible aging of the enzyme-OP complex can occur. Thus, the OP is continuously detoxified. Based on the two above observations, (a) that polyurethane foams are excellent adsorption materials for OPs such as pesticide vapors (6), and (b) that soluble ChEs and oxime together have the ability to detoxify OP compounds, we combined these features in a porous polyurethane foam formed *in situ* from water-miscible hydrophilic urethane prepolymers and the enzymes.

Detection of OP compounds is of paramount importance to prevent casualties due to OP exposure and cholinergic crisis. Traditional analysis of cholinesterase inhibitors is performed using gas and liquid chromatography and mass spectrometry (7). More problematic is the measurement of nerve agents in mixtures, which require extraction procedures and manipulations. These instrument intensive techniques have significant drawbacks when considering an individual kit for field deployment, including lack of portability, simplicity, cost, reliability, and rapid results. An alternate technology is a biosensor. Biosensors (8) have been widely used to detect biological, pharmacological, or clinically important compounds. Enzyme sensors have the advantage of selectivity, sensitivity and, most important, specificity, ease and portability, and markedly simplified instrumentation. Enzyme sensors can behave as a dosimeter, accumulating only those inhibitors demonstrating exquisite selectivity for the specific enzyme, while ignoring all other environmental interference. A variety of biosensors based on cholinesterases

immobilized non-covalently have been described (9). The drawback to these methods includes lack of enzyme stability at ambient conditions once opened, leaching from the surface to which it was non-covalently deposited, sensitivity to denaturing conditions, and short half-life when in solution. The currently fielded spot detector, the M256A1 chemical agent detector kit, has a dry eel ChE non-covalently applied (dried) onto a fiber: it can only be exposed to and monitor air or vapor for OPs. The major advantage of the immobilized ChE-sponge is that the enzyme will not leach from the polyurethane support so that the OP ticket or badge can be used to sample anything from soil, water, to air.

We envision a reusable immobilized enzyme sponge of cholinesterases and oximes for OP decontamination. Also, the immobilized polyurethane enzymes will make versatile biosensors for detecting organophosphates in all environments.

METHODS

Sponge synthesis and assay: The immobilized enzyme-sponge can be synthesized and cured in less than 20 minutes at ambient temperature, molded into the shape of any container. A new technique (10) was developed to mix the prepolymer (Hypol prepolymer TDI 3000, Hampshire Chemical, Lexington, MA) and enzyme in buffer containing 1% surfactant (Pluronic P-65, BASF Specialty Chemical, Parsippany, NJ). This method replaces the rapid mixing by an electric drill with a mixing stator (a stationary plastic disposable tube for two-component mixing) to effectively reduce high shear stress and partial denaturation of the enzymes during mixing of the two components, prepolymer and enzyme. In addition to simplicity and easy scale-up, the activity of ChEs coupled to the prepolymer increased about two-fold with the mixing stator compared to the high-speed drill mixing. The decontamination sponge containing the immobilized enzymes molded in a Tupperware® container the size of a human hand is shown in Figure 1 (left), while a sensor sponge, about 1 cm in diameter, is illustrated on the right. The sponges were evaluated for activity, such as temperature and environmental resistance, inhibition by OPs and reactivation by oximes, and stability using the appropriate technique for each enzyme, e.g., the modified Ellman method for ChEs.

RESULTS

Previously, we demonstrated (10) that immobilized ChEs showed little leakage from the sponges even after many washes over several days, and therefore were irreversibly and covalently incorporated into the polyurethane matrix. The enzymes attach covalently at surface lysines to the inert foam at multiple points during the polymerization process, thereby becoming an integral part of and acquire the structural integrity of the resulting polymerized matrix. The resulting ChE-sponge, a matrix of TDI polyurethane and enzyme, results in remarkable stability of these immobilized enzymes; ChE-sponges retained their original activity after more than 3 years at 0°C, most of their activity after 1.5 years at 25°C, and more than 50% activity after 1.5 years at 45°C.

Our recent results demonstrate the following additional characteristics of sponges containing immobilized AChE. We evaluated different polymers for immobilization of the enzymes. Originally, we used tolyl diisocyanate (TDI, 5%) and methylenediphenyl diisocyanate (MDI, 5%) polyether prepolymers. The TDI prepolymer proved more suitable to enzyme immobilization, presumably due to its flexible structure, and the TDI yielded ChE-PUFs with enhanced resistance to environmental denaturation. We have now evaluated prepolymers containing 3% TDI and 5% isophorone diisocyanate. The latter prepolymers yielded about the same efficiency for covalently coupling AChE and retained a high degree of esterase activity. We also describe here that the OPs diisopropylfluorophosphate or MEPQ (7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide) inhibited the activity of ChE-sponges, as was observed for non-immobilized ChEs in solution. The oxime HI-6 restored activity of the OP-poisoned AChE-sponge until the molar concentration of MEPQ reached approximately 1000 times that of the cholinesterase active site. However, the AChE-sponge could be recycled many times by rinsing the sponge with HI-6 in the absence of OP. In this case, most of the original cholinesterase activity could then be restored to the sponge. We now find that the ability of the immobilized enzymes and HI-6 to detoxify the OP MEPQ was dependent upon the efficiency of the sponge to decontaminate particular surfaces. The sponge alone could remove/decontaminate MEPQ from non-porous plastic surfaces (>97%), and an AChE-sponge with HI-6 completely detoxified the removed MEPQ. However, the sponge without enzyme was not more effective than the M291 decontamination kit for removing neat soman applied to guinea pig skin. We therefore evaluated additives to the polyurethane matrix, both during synthesis and post synthesis, to improve the removal and extraction of OPs from guinea pig skin. These experiments were performed with sponges lacking enzyme so that we could directly evaluate the ability of the sponge to decontaminate the skin. The prepolymer was not altered since currently there is no formulation of Hypol prepolymer with an increased hydrophobic nature that might be expected to absorb the OP more effectively. Liquid additives possessing surfactant properties, zwitterion and buffers, and partial organic solubilizing characteristics were tested, including centrimide, 18-Crown-6, iso-octane, kryptofix 222, polyethylene glycol 6000, triacetin, and tetraglyme.

We found that most solutions provided no significant benefit over the original phosphate buffer included in the sponge that was optimized for enzyme activity. However, both triacetin and tetraglyme provided additional ability to remove soman from the skin, protecting guinea pigs about four to five-fold better than the M291 kit (Table 1). In addition, sponges were synthesized so that activated carbon would be incorporated into the polymer matrix. The addition of carbon did not interfere with the immobilization of ChEs. However, while carbon-sponges alone provided mixed results for removing soman from skin, they might be effective in removing other toxic agents. Sponges containing 2-PAM and HI-6 also showed increased protection to soman skin toxicity compared to the M291 kit, at least four-fold (Figure 2).

We have also prepared small polyurethane badges/sensors approximately 1 cm in diameter in which the cholinesterases (FBS AChE and/or equine BChE) are covalently immobilized, as shown in Figure 1. The badges composed of mammalian AChE or BChE have similar kinetic properties to soluble enzyme and selectivity to OPs. Most important, the OP MEPQ inhibited either forms of the enzymes, soluble or immobilized, at the same rate as determined by their bimolecular rate constants (Table 2).

We have developed alternate indicator systems for the ChE badges: OP-exposed badges can be evaluated qualitatively by visual color changes or luminescence for dark-adapted eyes. The latter reaction uses a coupled reaction of immobilized ChE, choline oxidase, and peroxidase. Like the ChEs, choline oxidase was successfully immobilized to the prepolymer using the same mixing apparatus. We found that soluble and immobilized choline oxidase activity had similar kinetic parameters, and the pH dependent activity of the soluble or immobilized choline oxidase was identical. Since the optimal pH for ChEs and choline oxidase were the same (about pH 8), the coupled reaction in the assay could be simultaneously optimized for both immobilized enzymes. In another indicator reaction, we used Amplex Red, a reaction that generates an intense red color that is also fluorescent. The fluorescent chromagen yielded about a 4000-fold increase in sensitivity compared to the visible red chromagen. This fluorescent indicator would be particularly useful in a hand-held fluorometer, which could provide quantitative values of ChE inhibition instead of qualitative information.

One significant difference and advantage the immobilized enzyme sensor has over the current M256A1/M272 chemical agent detector kit is that immobilized enzymes do not dissociate (leach) from the sensor. Therefore, the immobilized enzyme sensor can be used to test water or even left in the liquid source for extended periods. To develop this concept, we prepared sensors using purified AChE and BChE and exposed them continuously to different pHs between 4-10.5 for over 2 months at 25°C. The badges showed good retention of enzyme activity and could remain at pH 6-8 in aqueous solution for more than 60 days. However, at the pH extremes of 4 and 10.5, the badges were sensitive to pH-induced inactivation, since a recovery period at pH 8 restored <20% of the original activity of the badge. It is known that both cholinesterases in solution are rapidly and irreversibly inactivated below pH 5 and above pH 9.5. Thus, immobilization affords only partial protection. In another situation, we placed the ChE sensors in natural water samples at room temperature (Figure 3, fresh water from the Allegheny River, PA). The sensors retained high activity even if the samples were not autoclaved, indicating that the immobilized enzymes were resistant to microbial degradation. In contrast, the M272 kit designed for water samples retained activity for minutes, rather than more than a month for the immobilized sensor (Figure 3). Therefore, the immobilized enzyme badges/sensors could be placed in the natural environment such as dirt to detect OPs, and then rinsed with local water to remove any interfering material before initiation of the color reaction to determine if OPs are present.

CONCLUSIONS

In conclusion, the capability to decontaminate personnel is extremely valuable to the military. The system must be lightweight, nontoxic to personnel, highly efficient, and compatible with sensitive medical areas such as eyes. In addition to decontamination of skin and personnel, the enzyme-sponges can be utilized for preventing cross-contamination of medical and clinical personnel. Still more uses for these formulations could include decontamination foams as masks and in garments, augmenting carbon filters that absorb OPs without inactivating them. OPs in the environment could be contained and decontaminated if the ChE-sponges were incorporated into fire-fighting foams. The enzyme-sponges could be used to decontaminate sensitive equipment without posing new environmental disposal problems, since the final products are rendered inert. Indeed, the sponges should be suitable for a variety of detoxification and decontamination schemes for both chemical weapons and civilians exposed to pesticides or highly toxic OPs such as sarin. This is important since currently accepted methods for decontamination of personnel, large areas, and materials use sodium hydroxide and bleach, which are caustic and harmful and also pose a significant environmental burden. In addition, the ChE-sponges could be used in chemical-biological sensors and incorporated into electronic hand-held telemedicine devices, for instance as electrochemical OP probes. The immobilized enzyme sensors have the unique ability, unlike the current OP ticket, to detect OPs in any environmental condition, such as vapor, water, or even soil, and in circumstances requiring long-term remote

sensing. Also, the immobilized sensors could be developed into a differential OP detector to identify the type of OP contamination, which would aid in treatment. With the constant threat of chemical warfare, terrorist acts, or spill of pesticides, the development of alternative means of protecting and decontaminating individuals from exposure to OP nerve agents is critical.

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KEYWORDS

Decontamination, Detoxification, Immobilized enzymes, Cholinesterases, Organophosphates, Biosensors

FIGURES AND TABLES

Table 1. Sponge Additives Protect Soman Contaminated Guinea pigs

Additive to sponge	**LD_{50}**	**Protective Ratio**
HI-6 (oxime)	79	8.0
2-PAM (oxime)	76	7.7
Tetraglyme	88	8.9
Reference Values		
M291 decontamination kit	17.7	1.8
Soman alone	9.9	

Table 2. Effect of Immobilization on ChE Kinetic Parameters: Time-Dependent Inhibition of ChEs by the OP MEPQ

ChE	**Enzyme Form**	**Bimolecular rate constant**
		(M^{-1} min^{-1}) ± SD
FBS-AChE	soluble	$1.59 \pm 0.52 \times 10^{8}$
	immobilized	$1.00 \pm 0.28 \times 10^{8}$
Equine-BChE	soluble	$4.15 \pm 0.78 \times 10^{7}$
	immobilized	$4.21 \pm 2.00 \times 10^{7}$

79. CHEMICAL DISASTER TRAINING FOR FIRST RESPONDERS

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ABSTRACT

Hazardous chemicals have been released during thousands of accidents, natural disasters, conflicts, and crimes over the past decade. First responders have usually been military personnel, disaster assessment teams, police officers, firemen, and emergency medical technicians. Decisions made by these first responders can have significant impacts on public health and safety. In response to requests from several United States Government agencies, the author developed a chemical disaster database and training course for first responders .

The database consists of information about chemical disasters likely to be encountered by first responders, as well as assessment factors and appropriate assistance actions for each type of disaster. Three key words (DISASTER, ASSESSMENT, and ASSISTANCE) were used as mnemonics to aid first responders in remembering critical disaster, assessment, and assistance factors. Each letter in a key word addresses a critical factor. Key words and factors are presented below.

DISASTER:
* Dates;
* Incidents;
* Sites;
* Acids and bases;
* Smoke and fire;
* Toxic substances;
* Explosives; and
* Reactive substances.

ASSESSMENT:
* Amount released;
* Source controls;
* Substances;
* Exposure pathways;
* Symptoms;
* Sensitive populations;
* Morbidity and mortality;
* Equipment available;
* Needs (e.g., shelter) and
* Training level of target audience.

ASSISTANCE:
* Acute care;
* Sequella and care;
* Information;
* Safety and security;
* Training assistance;
* Analyses (e.g., biomarkers);
* Necessities (e.g., food);
* Communications; and
* Equipment needed.

The database includes disasters involving mines, petroleum facilities, agricultural chemicals, chemical plants, bombing and arson incidents, poisoning incidents, clandestine drug laboratories, and environmental pollution. The course has been successfully used to train first responders in the United States about a broad range of potential chemical disasters.

INTRODUCTION

Almost 14,000 incidents involving emergency releases of hazardous chemicals into the environment occurred in the United States (U.S.) between 1987 and 1994.[1] Currently, about 1,000 chemical release emergencies are reported to the U.S. National Oil and Hazardous Substances Response Center each month. In the vast majority of cases, first responders on the scene were not physicians, toxicologists, or environmental health scientists with expertise in hazardous materials emergency response. First responders were, and are, almost always police officers, fire fighters, emergency medical technicians, and in some large metropolitan areas, hazardous materials technicians. The decisions they make can have major impacts on health and safety for themselves, other first responders, and the public. These first responders need concise, accurate, specific information about the chemicals involved in emergencies; assessment procedures for rapidly identifying the type and magnitude of hazards to public health and safety; and appropriate assistance actions to mitigate or prevent those hazards.

Other responders who may benefit from this type of information include corporate emergency response and security staff, international disaster response personnel, and staff from non-government organizations involved in providing relief to victims of chemical emergencies.

The Agency for Toxic Substances and Disease Registry (ATSDR) was created by the U.S. Congress in 1980 to provide a broad range of public health services related to human exposure to hazardous substances released into the environment.[2] These services include developing and maintaining an inventory of literature about public health effects of hazardous chemicals, and providing health education and technical assistance to other government agencies in the event of emergencies involving hazardous chemicals.

Occupational health and safety laws in the U.S. require persons exposed to hazardous chemicals to receive training prior to working in chemical environments.[3] However, many first responders have had little or no chemical

safety training related to their agency's specific mission.

In response to requests from several Federal, State, and local government agencies in the U.S., ATSDR developed pilot training courses and reference materials for those agencies. Unlike other reference materials, these were based on public health and safety information for types of incidents rather than on individual chemicals.

METHODS

ATSDR received requests for training from the following agencies;

- U.S. Department of Defense (hazardous chemicals in conflict zones);
- U.S. Agency for International Development (foreign chemical and radiation disasters);
- U.S. Department of Justice (hazardous chemicals related to law enforcement);
- State fire marshals in Nevada and West Virginia (chemical hazards related to arson);
- Sheriffs departments in Nevada and West Virginia (industrial chemicals used in bombs, arson, and poisoning incidents); and
- local police departments in several cities (chemical transportation accidents and chemical hazards related to clandestine drug laboratories).

ATSDR discussed training needs with each agency, then identified the types of incidents each agency addressed. The following types of incidents were included in a central inventory of information:

- chemical releases from mining activities (cyanide, mercury and other heavy metals, uranium);
- oil and gas industry fires, explosions, and chemical releases (pipelines, refineries, wells);
- chemical hazards related to natural disasters;
- fires, explosions, and releases of toxic substances from chemical industries;
- clandestine drug laboratory fires, explosions, and poisoning hazards;
- fire, explosion, and poisoning hazards related to nuclear and chemical weapons;
- pesticide poisonings;
- food, medicine, and beverage poisoning incidents (heavy metals, toxins, etc.);
- chemical and radiological transportation accidents (aircraft, rail, ship, truck);
- corrosive chemical assaults (throwing acid, abortion clinics and butyric acid);
- fire, explosion, and poisoning hazards related to bombing and arson incidents; and
- heavy environmental pollution hazards.

ATSDR then researched information about actual incidents to obtain real-world information about chemicals involved, health effects, assessment procedures, and appropriate assistance actions. In order to assist the memory of first responders, three key words were then developed, and relevant factors were assigned to each letter in each key word. The key words were DISASTER, ASSESSMENT, and ASSISTANCE. The key word DISASTER contained factors about dates, incidents, and chemical hazards in past emergencies to provide responders with historical information about each type of incident. The key word ASSESSMENT consisted of factors needed to conduct rapid, initial assessments of each type of incident. The key word ASSISTANCE included factors and sources of information to help responders rapidly identify appropriate initial assistance actions for the types of incidents they are most likely to encounter.

Key words and factors are presented below.

DISASTER:	ASSESSMENT:	ASSISTANCE:
* Dates;	*Amount released;	* Acute care;
* Incidents;	* Source and controls;	* Sequella and care;
* Sites;	* Signs;	* Information;
* Acids and bases;	* Exposure pathways;	* Safety and security;
* Smoke and fire;	* Symptoms;	* Training assistance;
* Toxic substances;	* Sensitive populations;	* Analyses (e.g., biomarkers);
* Explosives; and	* Morbidity and mortality;	* Necessities (e.g., food);
* Reactive substances.	* Equipment available;	* Communications; and
	* Needs (e.g., shelter) and	* Equipment needed.
	* Training level of target audience.	

An electronic database was developed consisting of worksheets for each type of incident. The worksheets were structured such that the titles for each record reflected a factor in a key word. Specific information needed to understand past incidents was extracted from reference materials into the factors in the key word DISASTER. Hazardous chemicals involved in each type of incident were mentioned under the factors entitled "Acids and Bases"(corrosive chemicals), Smoke and fire" (ignitable chemicals), "Toxic substances", "Explosives", and

"Reactives" (e.g., chemicals that are incompatible with other chemicals, oxidizers, and chemicals that polymerize violently with heat).

Information for the Assessment factors was obtained from peer-reviewed references, such as ATSDR's toxicological profiles,[4] ATSDR and Institute of Medicine Case Studies in Environmental Medicine,[5] and ATSDR's Medical Management Guidelines for Acute Chemical Exposures.[6]

In the Assistance key word, information about acute care and sequella, supplies, information sources, and communications (risk communication references) was obtained from textbooks on toxicology and poisoning,[7] the ATSDR references mentioned above, the North American Emergency Response Guidebook published by the U.S. Department of Transportation and partner agencies in Canada and Mexico,[8] and United Nations Environmental Programme Chemical Safety Cards prepared in partnership with the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention.[9]

Specific disaster incident information, assessment steps, and assistance actions were incorporated into records of the worksheets. The electronic worksheets were reviewed in accordance with ATSDR publications clearance procedures.

ATSDR then extracted worksheets containing information about types of incidents relevant to each client agency's mission and training needs. The worksheets were collated into training reference materials and distributed during pilot workshops. In addition, breakout sessions were developed based on local scenarios to provide the participants with opportunities to apply what they had learned to potential future disasters in small work-group settings.

The following workshops were conducted:

- International Chemical Disaster Module, U.S. Agency for International Development, Washington, DC, September 1998 and November, 1999 (30 participants, each workshop);
- Assisting Victims of Crimes Involving Hazardous Chemicals, 25th Conference of the National Organization for Victim Assistance, Los Angeles, California, September, 1999;
- U.S. Drug Enforcement Administration Victim-Witness Conference, Arlington, Virginia, February, 2000 (40 participants);
- Industrial Chemicals and Radiation in Conflict Zones, U.S. Department of Defense, Fort Bragg, North Carolina, February, 2000 (45 participants);
- International Ionizing Radiation Disaster Module, U.S. Agency for International Development, Washington, DC, February, 2000 (30 participants);
- Chemical Hazards Related to Law Enforcement, Law Enforcement Coordinating Committee, U.S. Department of Justice, Savannah, Georgia, March, 2000 (24 participants).

ATSDR obtained workshop evaluations from participants via independent sources. Participants were queried about the content, value, and organization of the material provided.

RESULTS

Results from the evaluations were positive from all organizations trained. In the initial workshops, one constructive critic recommended that the number of breakout sessions should be increased to provide participants with more opportunities to apply information being presented to them to future potential incidents. As a result, the number and scope of breakout sessions was increased from one per day to three or four per training day, and each session contained several different scenarios that the participants could address. Participants in subsequent workshops indicated that networking and the opportunity to think about applying key factors to future potential incidents were some of the most valuable aspects of the course.

In general, the positive evaluations indicated that a single repository of information, based on types of incidents, can be used to provide valuable training to first responders from a broad range of organizations. A central repository and database can also be used as an inventory of information about public health and safety effects from a broad range of emergencies and accidents involving hazardous chemicals.

Another result was that the incident-based research indicated that additional public health and medical publications were needed to address mixtures of chemicals at clandestine drug laboratories and improvised explosives manufacturing sites. Most of ATSDR's current Medical Management Guidelines for Acute Chemical Exposures, Case Studies in Environmental Medicine, and Toxicological Profiles were based on chemicals commonly encountered at hazardous waste disposal sites, chemical transportation accident sites, or industrial facility accident sites. ATSDR is currently evaluating a list of potential chemicals for development of additional Medical Management Guidelines.

CONCLUSIONS

Several conclusions can be drawn from the research and workshops.

1. First responders in chemical emergencies usually consist of police officers, fire fighters, and emergency medical technicians; not physicians, toxicologists, and environmental health scientists.
2. These professionals respond to incidents, not to individual chemicals.
3. Incident-based reference materials can provide guidance that "ties together" information about specific chemicals.
4. First responders want specific information, not more information. Concise references are most valuable.
5. Information for first responders should be written on the high school level.
6. A central, incident-based repository of information can be used to tailor training for a broad range of first responders.
7. Networking, thought, and discussions during breakout sessions based on plausible future potential incidents are some of the most valuable aspects of chemical emergency workshops for first responders.
8. Information about incidents and feedback from workshops can be used to identify areas for development of additional chemical-specific reference materials to safeguard public health and safety.

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KEYWORDS

Chemicals, disasters, emergency, training, database

80. THE ROLE OF POISON CONTROL CENTERS AND MEDICAL TOXICOLOGISTS IN RESPONDING TO CHEMICAL AND BIOLOGICAL INCIDENTS.

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The occurrence of a chemical or biological agent exposure incident in a locality will place tremendous demands on its medical and public health resources. Poison Control Centers and their affiliated medical toxicologists represent existing components of the public health infrastructure that are ideally suited to augment local response. The approximately 60 certified Poison Control Centers in the United States currently provide health professionals and the general public with telephone consultation on the assessment and management of poisoning emergencies 24 hours a day, 365 days a year. Affiliated with each center are one or more of the nation's approximately 200 medical toxicologists, physicians with specialized clinical training in the diagnosis and management of human poisoning. Because of their existing operations, Poison Centers and affiliated medical toxicologists have the skills necessary to rapidly differentiate potential CB poisoning incidents from non-emergent situations, and also have experience in use of atropine, oximes, and related agents in the treatment of accidental poisonings from organophosphate pesticides. In the event of a CB incident, Poison Centers can provide the following key services: a) centralized, knowledgeable communication to hospital emergency department personnel regarding triage and management of casualties; b) assistance in localization and deployment of antidotes and related emergency medical supplies; c) communication of information to the general public, either through the media or through incoming telephone calls, that may avert unnecessary utilization of emergency facilities by individuals who do not require immediate medical attention.

Keywords: poison control centers, medical toxicologists, triage

81. HISTORY AND EVALUATION OF THE US BIODETECTION PROGRAM

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INTRODUCTION

The US BW program began in April 1943, with the construction of the BW laboratories at Camp Detrick. The overall US BW program continued until 25 November 1969 when President Nixon renounced the first use of chemical warfare and renounced the use of all methods of biological warfare. Further, he directed the disposal of the entire stockpile of BW weapons and agents. He declared that all BW programs would be only defensive in nature: this included immunization, safety, and detection and warning programs.

The initial emphasis of the early US BW program was on agent delivery capabilities for anti-personnel and anti-crop agents. The Defensive Program, initiated at the same time, included safety, physical and medical protection, but the emphasis was always on safety. Since leaks of agents presented a real hazard to operating personnel and the community at large, the Camp Detrick Safety Division was assigned to detect microorganisms as quickly and as accurately as existing technologies allowed. This meant using traditional methods of bacteriology for the "rapid" detection of organisms: using the swab and streak method, as well as by sample inoculation into animals.

The detection and warning program efforts started in earnest in 1948. The effort was based on the requirements that detection and warning must be: agent specific; as rapid as possible; capable of detecting small numbers of organisms; and capable of handling large numbers of samples. Detection techniques were divided into direct observation of the aerosol and observation after its collection by using particle size analyzers, infrared spectroscopy, and microbiological techniques in metabolism, immunology, and enzymology.

PROBLEM

For BW purposes, the most effective and efficient route of entry into the human body is through the lungs. Aerosol particles in the 0.5 - 5 micrometer range, are the method of choice for delivery to avoid filtering by the nose and upper respiratory tract and to assure deposition into the lungs, causing foci of infection (1).

The US Public Health Service in the 1950's gathered air samples from all over the US and Alaska and determined that the average particulate concentration background in air was 100-300 micrograms per cubic meter of air (1 - 3 x 10 E -7 grams per liter of air). The average background of protein in the air was measured to be about 10 E-8 grams per liter of air. The average bacterial content of the air was determined to be approximately 2-4 organism per liter (2).

DISCUSSION

There are two general methods for the detection of a Biological Warfare Agent Aerosol: direct and indirect. In the direct method, the suspected agent aerosol is observed directly to determine particle size, infrared (IR) absorption, ultra violet (UV) absorption, or microwave absorption, etc. In the indirect method, a sample from the suspected agent aerosol is collected and concentrated before any examination. The observations made or tests done are in terms of viability/non-viability and general/specific. General observations include particle size, particle loading, protein content, and some metabolic reactions; specific observations and tests include metabolic reactions, immunological reactions, enzymatic reactions, antigen-antibody reactions, intrinsic fluorescence, chemiluminescence, bioluminescence, nucleic acid amplification, etc. (2).

Microwave Spectroscopy: This method measures the energy emitted by molecules as they undergo rotational and vibrational transitions induced by absorption of microwave frequencies. This method is claimed (2; 3) to have a 10,000 fold better discriminating capability than infrared spectroscopy; the spectral lines are very sharp and this allows precise determination of their frequency. However, samples are usually required to be in the gas phase and at low pressure for analysis. Early work used broad band microwaves and the results were determined to be due to local heating of intracellular water. With narrow band radiation and lower power there were real effects that could occur at specific frequencies; these transitions were mainly due to dipole rotations and architectural and positional effects of masses and charges. Although clearly different spectra due to DNA and RNA have been seen, it is doubtful that this technique is useful at atmospheric pressure because of the spectral line broadening. Pyrolysis of the sample and analysis at low pressure is a possibility, but there is no indication of any further work on this technique.

Infrared Spectroscopy: This method is based on the absorption of energy by structural groups in a molecule; absorption occurs at specific frequencies corresponding to vibrational and rotational transitions (4). The chemical composition of bacteria is so similar that their IR absorption spectra are very similar although the ratios of certain peak heights have

been used to differentiate species (2). However, the composition of the culture and method of treatment have to be uniform in order to obtain reproducible results. (Bacteria are what they eat).

A number of sample preparation techniques were investigated, such as gas chromatography, pyrolysis, etc. It takes between 90-100 micrograms dry weight of bacteria to get an interpretable spectrum as a standard, but even with computer assisted analyses, it remains a difficult job. IR spectroscopy is difficult to perform on wet samples, because water absorbs broadly across the infrared range. Stand-off use of IR is difficult because at least 10 E5 particles/liter are necessary for detection; the spectrum from 9 to 11 micrometers is essentially featureless and there is backscattering from naturally occurring aerosols (5).

Ultraviolet Spectroscopy: This method is based on the absorption of energy at specific frequencies corresponding to electronic transitions in the sample; the specific absorptions are characteristic both of the atoms present (the chromophore) and the chemical structure of the sample (the electronic environment) (4). Nucleic acids absorb strongly at 265 nanometers (nm); initial calculations indicated that UV absorption could detect bacterial cells at 2 organisms/L, although a high volume sampler and concentrator were required (6; 7). The amino acids tryptophan and tyrosine absorb strongly at 280 nm; however, they are merely indicative of proteins, rather than a specific organism (8). The nucleic acid absorption was proposed to distinguish airborne bacterial from dust particles, which generally have either a weak absorption at 265 nm or a strong absorption at 265 nm and a strong absorption at 312 nm, which is not present in nucleic acid (7). One of the major drawbacks found was that as cell cultures age to 18-24 hours, the absorption at 265 nm decreased significantly in intensity (7).

Using UV as a remote sensor was investigated, using an excitation-emission system. Tryptophan absorbs strongly at 280 nm and fluoresces at 350 nm (5). Prototypes were built and tested; although they worked, there was significant interference from hydrocarbons in the air. Additionally, because they detected only protein and not specific BW agents, their utility was lessened.

Staining Techniques: A variety of dyes or other materials were used to react with various organisms or specific structures; the results were then observed under a light microscope. The dyes and stains used included Gram stain, radioactive iodine modification of Gram stain, fluorochrome stain, biuret reaction, radioactive tungstate, phosphomolybdate, colloidal gold, resazarin dye, tetrazolium, and others. Staining is a time-honored method of identification of bacteria; however, these methods were judged not to be sufficiently specific or sensitive (6; 2).

Optical Rotatory Dispersion: Optically active molecules rotate linearly polarized light into right and left handed beams that have different refractive indices. Since the refractive index depends on wavelength, different wavelengths are rotated different amounts. The resulting spectrum describes the optical activity. Amide bonds in proteins are optically active, as are carbohydrates. Intact bacteria exhibit optical activity in the near UV and early experiments showed that species could be clearly differentiated. However the quantity of material required was 10 E8 - 10 E9 cells, so this technique was abandoned as not sufficiently sensitive (2; 4).

Particle Methods: Particulate matter is always present in the atmosphere, varying in size and concentration depending on the time of day, weather, geography and season. This particulate matter is both living (bioaerosols) and dead (dust, etc.) and is what is known as a normal background (9). The purpose of the particle method is to recognize an unusual increase in the number of particles in the size range 1.0 to 3.5 micrometers in the presence of the normal background. This size is specific for maximum retention in the lungs (1).

Single Particle Light Scattering: Single Particle Light Scattering is a concept based on light scatter from single particles, which is detected by a photomultiplier tube. A non-specific and very sensitive technique, it is capable of counting single biological or toxin particles. Background and man-made aerosols are differentiated based on determination of particle size and shape. Two light scattering single particle counters were built in the 1960s; they were configured as detectors and included alarm logic. The devices, which sampled only 0.2 liters of air per minute, were called Ratio Alarms. The logic was based on measuring quantities in two size ranges, which were then compared - one served as a reference size range and the other as the analytical size range. The bacterial aerosol particles were expected to cause an increase in the analytical size range with no change in the quantities in the reference range. The counting levels in the reference and analytical ranges prior to arrival of the bacterial aerosol was established as background. When the aerosol arrived, the device measured a change in the quantities in the analytical size and not in the reference size. The change in the ratio of the counts occurring in the two size ranges served as a detection device.

The minimum sensitivity of approximately 300 particles per liter was unacceptable The major drawback was the device's lack of specificity, which led to a high false alarm rate. The approach was abandoned in the early 1960's (5).

Particle Size Analyzer: The Particle Size Analyzer is a device for sizing and counting particles in the micron and sub-micron size range (0.3 to 2.5 micrometers). As they pass through a small, highly illuminated optical volume, the

particles are counted and sized by electronic observation of electrical pulses generated from forward scattered light (Mie scattering) falling on a photomultiplier tube (9). A ratio alarm system comparing two different channels, one the 1.0 - 1.5 micrometer size, was developed to give an alarm when there was a change compared to a smaller size such as 0.5 - 0.6 micrometers. A 10-15 % change in the alarm channel size was sufficient to give an alarm in the presence of a fluctuating background. However, this is generally a non-specific method, although it makes a good front end for more specific detector systems.

Partichrome Analyzer: This technique for detection was based on differentiation of bacteria from non-bacterial (e.g., dust) particles by staining collected particles with ethyl violet at 85o C, followed by destaining with acetone or another solvent. Vegetative cells and spores are both stained an intense blue, while other particles do not take up the stain to any extent (5).

Bacterial detection is a continuous process; Cronar tape is used to impact airborne material smaller than 5 micrometers, followed by staining and destaining, and then analysis. Approximately 2% of the non-bacterial particles stain blue, which give a false positive; further, only 75% of bacteria were stained. Pollen, which usually occurs in sizes larger than 15 micrometers, was removed by impaction and was not an interferent. Some naturally occurring biological materials that were not removed by impaction produced false alarms. Vegetative cells and spores in the size range of 1-2 micrometers had a background of 10-40 blue counts per liter at the instrument sampling rate. Critical focus and light source requirements, as well as complex optics were key problem areas. In addition, liquid reagents caused a logistics burden.

Fluorescent Antibody Staining Technique (FAST): In the FAST technique, antibodies to various organisms were tagged with a fluorescent material to form a fluorescent antibody which, when reacted with the appropriate antigen, yielded a specific fluorescent antigen-antibody complex. The fluorescent particles remaining after washing could be counted when illuminated with ultraviolet light.

The FAST technique was based on the impaction of a dry BW aerosol on a transparent tape, followed by applying suitable reagents, washing, illuminating and counting using an image plane scanning system. The principal difference between FAST and Partichrome is that FAST uses a fluorescent antibody stain, while Partichrome uses conventional microbiological stains. Two breadboard devices demonstrated a sensitivity of 2 organisms/liter of air with a normal background. This included detecting both spores and vegetative cells in the presence of background contaminants; the response time was 4-7 minutes.

The key problem with the FAST was its specificity, since a different antibody had to be prepared for each known organism. Other drawbacks were the requirement for liquids, which were difficult to store, and non-specific binding. Although FAST could detect a single bacterium, it could not detect viruses. The program was ended in 1964 (5).

Radioactive Antibody Staining Technique (RAST): This technique was based on the use of radioactively tagged antibodies. The radioactive label used was S35-sulfanilic acid. After the reaction occurred, the material was filtered and counted by a Geiger Muller tube or similar device. The extrapolated detection sensitivity was between 0.01 and 0.4 cells per liter of air in 10 minutes, with excellent opportunities to reduce the detection time to 5 minutes or less. RAST, like FAST, required a separately labeled antibody reagent for each biological agent that might be encountered. Like FAST, the RAST had a specificity problem. Drawbacks to RAST were the radioactive waste problem and the rather large sample size required. A comparison of RAST to FAST concluded that FAST was preferable to RAST for BW agent detection. RAST was dropped in 1965 (5).

Chemiluminescence: The principle employed in chemiluminescence detection of bacteria is based on the oxidation of luminol (5-Amino-2, 3-dihydro-1, 4-phthalazinedione) in an alkaline solution. Oxygen is provided by the catalytic decomposition of sodium perborate. The catalyst is porphyrin or hematin, provided by the bacteria. The total light emission is directly proportional to the concentration of the porphyrin-containing material.

The reaction produces light extending over 350-600 nm, and is analyzed by a photomultiplier tube with a sensitivity of 10 E4-10 E5 bacterial cells per milliliter, depending upon the particular organism. Hematin is used as a standard. A chemiluminescence device, assigned the experimental number XM19, was developed. The program was terminated in 1983 because the developed system was not sufficiently sensitive (5).

Mass Spectroscopy (MS): In this analytical technique, materials to be analyzed are converted into gaseous ions, fragmented and the fragments separated by their mass-to-charge ratio. MS is a highly specific, sensitive, and rapid analytical device. Many compounds, including peptides, nucleotides, and other polar or high molecular weight substances, originally were uncharacterizable by this technique because they could not be volatilized without significant degradation. Sample preparation techniques such as Curie-point pyrolysis, plasma desorption and laser desorption, allow MS to be applied to many more types of samples than previously, including intact biological organisms. (2)

One problem with MS as a direct analytical tool for intact organisms is the tremendous amount of data; the fragmentation pattern is overwhelming. The spectra are almost uninterpretable. In one approach to limit the spectrum,

bacterial samples are pyrolyzed, then analyzed by gas chromatography/mass spectrometry (10). Other methods include tandem MS, in which specific fragments are further ionized. MS is under investigation and development for use as a BW agent detector/identifier by the US.

CONCLUSION

This has been a brief summary of some of the methods examined by the US for detection of BW agents. Some were good laboratory methods, some were not sensitive enough. BW agent detection and identification in real time is a continuing problem and is the subject of much research.

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83. THE DEVELOPMENT OF BOTH LABORATORY PROTOCOLS FOR IDENTIFYING BIOTERRORIST THREAT AGENTS AND A NATIONAL NETWORK FOR INFORMATION DISSEMINATION TO PUBLIC HEALTH LABORATORIES IN THE UNITED STATES

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INTRODUCTION

In order to strengthen public health laboratory infrastructure, the Bioterrorism Preparedness and Response Program (BPRP) at the Centers for Disease Control and Prevention (CDC), in conjunction with other key partners developed laboratory protocols for rapid identification of critical biological agents (e.g., the causative agents of anthrax, plague, tularemia). Through a cooperative agreement with public health laboratories, BPRP distributed 43 awards to state and local public health laboratories in order to strengthen the capacity of these laboratories to rapidly identify critical biological agents. BPRP continues to provide technical assistance to the network laboratories, and will soon offer advanced wet laboratory training. The bioterrorism preparedness and response funding and training initiatives will enable public health laboratories to participate in a national response network in the event of a bioterrorist attack. This nation-wide emergency preparedness and response process to an act of bioterrorism depends upon immediate access to web site-ready critical biological agent identification laboratory protocols.

The evolution of developing both the laboratory protocols and the restricted web site to distribute critical information during a bioterrorist attack is an important component of the national preparedness and response plan. Here, examples of basic versus advanced procedures are given, a pyramid of the laboratory response network identified, and a discussion of lessons learned from the rapid start-up of the laboratory response network. These are brief glimpses into the CDC initiative that focuses on strengthening the laboratory capacity of the public health sector to respond to a bioterrorist incident. Moreover, there is an added benefit of enhancing public health responses to emerging infectious diseases and to outbreaks of unknown disease.

MATERIALS AND METHODS

Laboratory protocols: development of laboratory protocols include the provision of technical content by subject matter experts from CDC, United States Army Medical Research Institute of Infectious Diseases (USAMRIID), and public health laboratory scientists. Legal and forensic contributions to the protocols were made by federal law enforcement officials. The laboratory reagent production was designed through a collaborative effort which included the Scientific Resource Program at CDC, as well as other state and federal partners.

Web site development: the development of the private and public web was a collaborative effort between the Association of Public Health Laboratories, state, federal, local organizations, and the Health Alert Network within the Public Health Practice Program Office at CDC.

RESULTS AND DISCUSSION

Working from the critical agent list, a team consisting of representatives from state public health laboratories, subject matter experts, Department of Defense, CDC/BPRP, and federal law enforcement identified which tests would be best to use in the event of a bioterrorist attack. Direct fluorescent antibody assays were chosen as they are based on a technology in which state/local public health laboratories were experienced. Additional assays were chosen based on the need to have viable organisms or on specimen type. Once the protocols were developed, potential commercial sources for reagents were identified. CDC obtained and inventoried the reagents if there were no commercial alternatives. Reagent ordering and profile information is available on the web site of the Laboratory Response Network.

Basic and advanced laboratory procedures (9-10) for identifying the critical biological agent Francisella tularensis are shown in Table 1. These procedures range in difficulty and safety requirements. For example, F. tularensis growth characteristics in agar or broth can be performed in a biosafety level 2 laboratory (11), working in a Class II biological safety cabinet. Whereas, immunohistochemistry identification (12) of F. tularensis is done in a biosafety level 3 laboratory (11). The laboratory procedures

(e.g., growth characteristics in broth) are available on-line at www.bt.cdc.gov. The advanced capacity pubic health laboratories (refer to Figure 1) can participate in various studies, such as validation of new assays and reagents. Rapid tests suggested by public health laboratories are added to the protocol manual and updated on the web site, constituting a "living" laboratory manual. Plans exist to add TaqManTM chemistry nucleic acid amplification techniques in the near future. The Bioterrorism Preparedness and Response Program is dedicated to providing training and web site-ready laboratory protocols for use with rapid diagnostic equipment to identify an agent used in a bioterrorist attack.

The lessons learned from the rapid start-up of the laboratory response network include; the need for better communication between the epidemiology and laboratory divisions of public health laboratories, regulatory concerns when rapid approval of identification techniques become diagnostic determinants in patient management, informed consent issues, and private companies not wanting to participate once the word "bioterrorism" is used in conjunction with a particular test. There have been lessons learned in trying to coordinate cooperative agreements and communication with the epidemiology and laboratory sections of a public health laboratory. At times, we have made assumptions that these 2 sections of a laboratory are communicating when in fact they are not. This disconnect in communication has led to several problems such as, epidemiology sections that call federal law enforcement without having their own laboratory section do a rule-out test on an organism. The antithesis occurs as well, where a laboratory section of a public health laboratory tests a sample, records what agent it was, and does not communicate with the epidemiological surveillance section that is tracking an epidemic. The bioterrorism preparedness and response laboratory team learned many lessons from the rapid start-up of the program, strengthening of the public health laboratory infrastructure, and implementation of a national laboratory response network.

SUMMARY

The bioterrorism preparedness and response laboratory protocol development process at CDC involves content contribution from subject matter experts, and forensic contributions from law enforcement agencies. The protocols outline the role of public health laboratories during a covert or overt bioterrorist event and provide detailed laboratory procedures, and the reagents to perform those procedures. In order to effectively disseminate the laboratory protocols to public health laboratories throughout the U.S., CDC collaborated in the development of a laboratory response network. This paper describes the development of both the laboratory protocols and the web site used to distribute critical information for rapid responsiveness to bioterrorist attacks. The laboratory protocols drive the entire process, including reagent production, vaccine discussions, safety workshops, and web site development. New critical agents are added to the list annually, and it is imperative that U.S. public health laboratories have the tools (protocols and reagents) to identify the agents, so that necessary medical intervention can be rapidly implemented.

The lessons learned from the development of the laboratory response network are an end result of putting together a program of individuals that planned, managed, and produced a rapid start-up network. This involved; judging capacity of laboratories, providing funds, getting the necessary representatives to a cooperative forum (e.g., subject matter experts, various scientific advisors, federal law enforcement, information technology experts, logistical managers, and public health laboratory directors from various states), determining what tests to use, partnering with multiple agencies and associations to produce protocols, reagents, cooperative agreements or contracts for informatic solutions, and working out policy agreements with federal organizations. The initial start up of the program began in January, 1999, and needed to move quickly in order to stand up a public health network system within 8 months. Our goal to rapidly strengthen the public health infrastructure led us to our current point as a national response network of public and federal laboratories capable of responding to bioterrorist attacks.

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KEYWORDS
Bioterrorism, Laboratory Protocols, Emergency Preparedness, and National Response Program.

FIGURES AND TABLES

Figure 1. Pyramid showing the various levels of the laboratory response network.

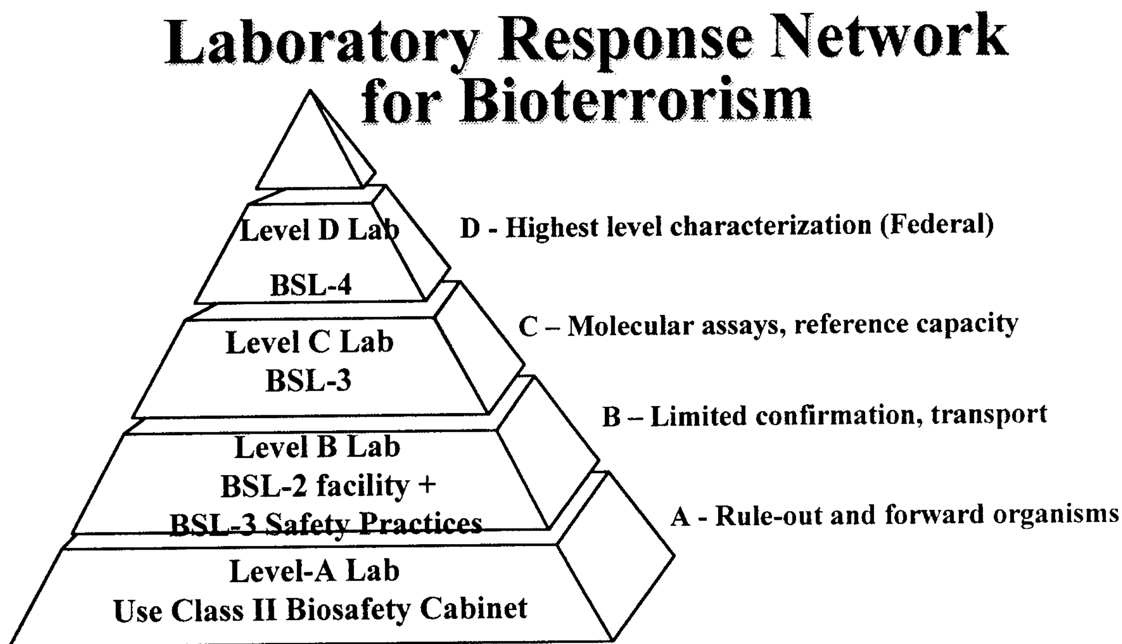

Table 1. A comparison of laboratory procedures for identifying *Francisella tularensis*

Basic Procedures:	Advanced Procedures:
Gram Stain	Immunohistochemistry
Growth in Broth	Environmental Specimen Evaluation
Growth in Agar	Development of New and Improved Tests

84. CHOLINESTERASES, OXIMES, AND ORGANOPHOSPHORUS HYDROLASES IN TANDEM CAN HYDROLYZE ORGANOPHOSPHATES

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Reactivation of organophosphate (OP)-inhibited acetylcholinesterase (AChE) is a key objective in the treatment of OP poisoning. Reactivation is accelerated by ligands such as edrophonium and decamethonium which may be due to the protection of regenerated enzyme from inhibition by the putative product, the phosphoryloxime. Using recombinant wild-type and D74N mouse AChEs, each inhibited with MEPQ (7-(methylethoxyphosphinyloxy)-1- methylquinolinium iodide) and DEPQ (7-(O, O-diethyl-phosphinyloxy)-1-methyl-quinolinium methylsulfate), a relationship was established between ligand-induced acceleration of reactivation and phosphoryloxime inhibition of the reactivated enzyme. Results showed that phosphoryloxime inhibition occurs during reactivation by pyridinium oximes LüH6 and TMB4, and not with HI-6. This is due to the extreme lability of the phosphoryloximes formed by HI-6. Phosphoryloximes formed during reactivation of ethoxy methylphosphonyl-AChE conjugate by LüH6 and TMB4 were isolated and their structures confirmed by ^{1}H NMR. The purified phosphoryloximes formed with LüH6 and TMB4 are 10- to 22-fold more potent than MEPQ as inhibitors of AChE and stable for several hours at pH 7.2 in HEPES buffer. However, phosphoryloximes formed during the reactivation of diethylphosphoryl-AChE conjugate were not sufficiently stable to be detected by ^{31}P NMR. Reactivation of both ethoxy methylphosphonyl- and diethylphosphoryl-AChE by LüH6 and TMB4 was accelerated in the presence of rabbit serum paraoxonase, suggesting that organophosphorus hydrolase (OPH) can hydrolyze phosphoryloximes formed during the reactivation reaction. These results suggest that ChEs, oximes, and OPHs can work in tandem to hydrolyze or inactivate OPs *in vivo* and *in vitro*.

Keywords: phosphoryloxime, cholinesterase, organophosphorus hydrolase, oxime, reactivation

85. BIOCIDE PROGRAM: MEDICAL PRODUCTS FOR BIOLOGICAL WARFARE DEFENSE

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INTRODUCTION

Under U.S. Army sponsorship, the Biocide Program has brought together Battelle Memorial Institute, Gentex Corporation, and Hydro Biotech to develop unique medical biological defense products. A novel biocidal material (Triosyn®) produced by Hydro Biotech of Montreal Canada has been adapted to prototype products developed in cooperation with the Medical Research and Material Command and the Soldier, Biological and Chemical Command.

MATERIALS

Triosyn® biocidal resin is an interactive broad-spectrum biocidal polymer effective against bacteria (vegetative and spore), virus, protozoa, algae, and fungi. Triosyn® interacts with microorganisms by an ion exchange mechanism. The presence, type and population density of the microorganisms activate the ion exchange mechanism. Triosyn® incorporates a complex iodine molecule that releases I_2, which reacts with the organism creating the demand. This demand release property of Triosyn® makes it superior to iodophors and tinctures of iodine, in that less iodine is released into the environment.

Efficacy testing of Triosyn® under various conditions was conducted at Hydro Biotech and Battelle, as well as other independent laboratories. In each of the tests, Triosyn® was shown to be effective in killing microorganisms. The results were dependent on the ability of the system to insure that the Triosyn® and microorganisms come into contact.

RESULTS AND DISCUSSIONS

A canteen with a self-contained filtration system, which is compatible with the existing U.S. military cup and cover, has been developed. It allows users to collect, purify, and drink water that would otherwise be unsuitable for consumption. The combination filter is composed of a ceramic body with Triosyn® enclosed in the core. The filter purifies the water supply as it is pumped into the canteen. The ceramic portion removes particulates greater than 0.2 microns and the Triosyn® inactivates microorganisms. When the ceramic body becomes clogged it can be cleaned and the filter remains useable for approximately 100 gallons of water.

The major advantage of Triosyn® over existing water treatment systems is that Triosyn® releases iodine on demand rather than creating a solution with iodine, thereby reducing the quantity of iodine used for each application and minimizing the residual iodine taste.

Results obtained from initial testing of the filter unit exceeded the reduction requirements for *Klebsiella terrigena* (6 logs) and MS2 (4 logs) set by the EPA Guide Standard and Protocol for Testing Microbiological Water Purifiers.

The prototype battlefield casualty care and evacuation system features stays to hold the enclosure away from the victim, windows to view the patient, glove ports for medical intervention, oxygen and IV ports, a full-length zipper for entry/exit, and an equipment pass through. Additionally, this care system has carrying handles and attaches to all existing litter systems. The core material is permeable and possesses inherent protective properties against biological and chemical warfare agents. The Triosyn® layer will protect medical personnel and patients from a biohazard and the chemical protective layer will protect individuals from a chemical threat.

The major advantage of this casualty care system is that it will allow medical personnel access to patients while protecting both the staff and patients from contaminants that may transfer into or out of the care system. The current chemical casualty bag does not offer medical staff access or biohazard protection. The blowers provide filtered airflow directed through the system as dictated by the status of the patient while providing increased patient comfort.

An additional item of equipment has been designed for use with the currently fielded body bag liner. This kit is comprised of an aerosol can of Triosyn® and a closure tape. The remains are placed in the bag liner; the bag is zipped almost closed; the remains are sprayed with Triosyn® to decontaminate exposed surfaces; and the closure tape is use to seal the zipper. The Triosyn® laminated fabric patch in the closure tape allows the bag to pressurize when transported by air.

Use of these items will provide improved containment of infectious remains and reduction of biological hazard on those remains. Because of this, the personnel contacting this modified body bag are protected better than with the existing bag alone. The liner remains compatible with the existing outer bags in the U.S. military inventory.

SUMMARY

A number of medical protective measures have been developed to protect against biological warfare threats. The basic technology involves a novel biocidal material Triosyn® which releases iodine on demand when it comes in contact with microorganisms. The design of the medical materials has been accomplished through a cooperative effort of three industrial partners and Department of Defense scientists, engineers and planners. The uses for this technology and its applications extend beyond the military and are applicable to civilian and medical needs.

KEY WORDS

Biological warfare, decontamination, water purification, iodinated resin, casualty care

FIGURES AND TABLES

Figure 1. Canteen and self-contained water filter/purifier

Figure 2. Casualty care and evacuation system

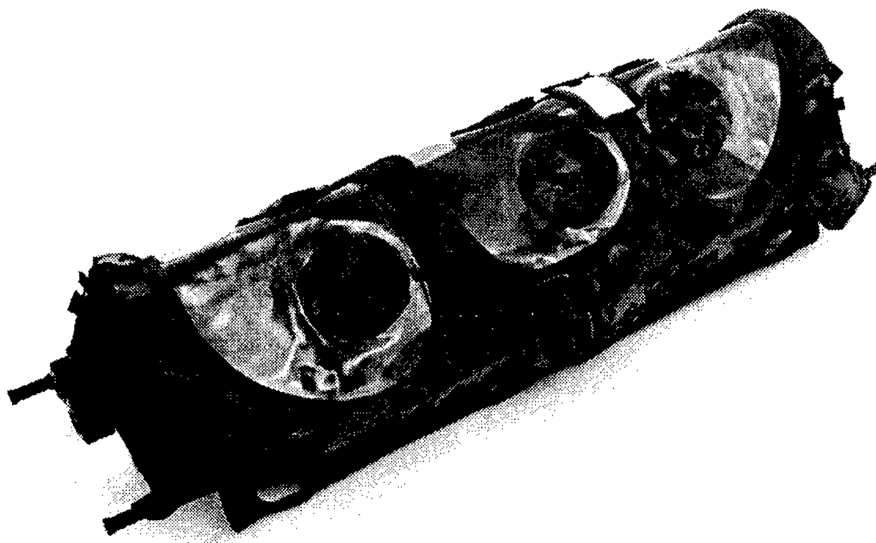
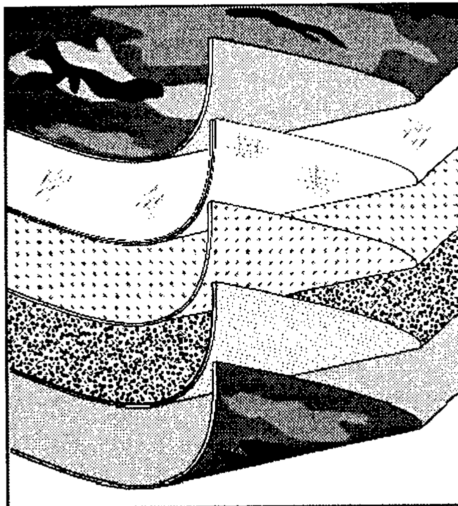


Figure 3. Laminate structure of the Casualty Care System

CASUALTY CARE SYSTEM
LAMINATE STRUCTURE



CAMO TWILL

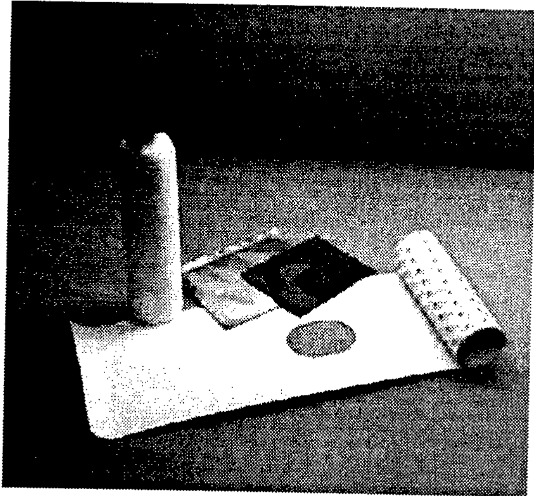
GENTEX CD2610 CHEM
PROTECTIVE MATERIAL

CHARGED MELTBLOWN
FILTER MATERIAL

TRIOSYN LAMINATE BIO
PROTECTIVE MATERIAL

CAMO TWILL

Figure 4. Body bag and spray can.



86. DIISOPROPYLFLUOROPHOSPHATE (DFP) ANTAGONISM BY RECOMBINANT ORGANOPHOSPHORUS ACID ANHYDROLASE (OPAA) ENCAPSULATED WITHIN STERICALLY STABILIZED LIPOSOMES (SL)

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INTRODUCTION

The investigation was focused on antagonizing organophosphorus (OP) intoxication by employing a new conceptual approach, which is based on encapsulation of recombinant enzymes within biodegradable enzyme carrier systems. OP compounds exert their toxic effects by inhibiting acetylcholinesterase (AChE) causing an excessive accumulation of the neurotransmitter, acetylcholine, and subsequent disruption of cholinergic nervous transmission. Clinical therapy presently uses the OP antidotes atropine and 2-PAM. Atropine antagonizes acetylcholine at the muscarinic sites, and pralidoxime (2-PAM) reactivates the OP inhibited acetylcholinesterase (1). But neither 2-PAM nor atropine degrades the OP agent. One of the fundamental mechanisms for the detoxification of OP compounds involves the degradation with OP-hydrolyzing enzymes so that the OPs are unable to phosphorylate the AChE. Using exogenous enzymes in OP antagonism previously has been reported (2), (3) and (4). However, the injection of purified free enzyme preparations into the blood stream has serious limitations because of immunologic reactions and various adverse physiological disposition factors. Biodegradable liposome carriers, which are permeable to the toxin molecules, can efficiently provide large amounts of the highly purified metabolizing enzymes to remain in the circulation for long periods of time. Carrier resealed annealed erythrocytes (CRBC) were first used as drug/enzyme carriers 2-3 decades ago (5) and (6). Drugs and enzymes were encapsulated into resealed annealed red blood cells by hypotonic dialysis. CRBCs were successively employed as carriers with rhodanese in cyanide antagonism (7), (8), (9), (10), (11) and (12) and in OP antagonism (13) and (14). Encapsulation of squid-type diisopropylphosphorofluoridate-hydrolyzing enzyme (DFPase) into carrier red blood cells was first reported in 1993 (15). Sterically stabilized liposomes have been widely used as targeted drug delivery systems in the clinical therapy (16). In the stealth liposome system, a drug is encapsulated in a biocompatible carrier vesicle that can circumvent the body's immune defenses, thereby circumventing rapid uptake by the macrophage cells of the reticuloendothelial system (16), (17), (18) and (19). Previous studies reported the antidotal effects of OP hydrolase (OPH) when it was encapsulated within sterically stabilized liposomes (20). OP hydrolase (OPH) and OPA anhydrolase (OPAA) have different substrate specificity. OPH hydrolyzes paraoxon and other agricultural insecticides and OPAA degrades DFP, sarin and soman (21). OPAA is a prolidase enzyme, which can hydrolyze dipeptides with a prolyl residue at the carboxyl terminus (E.C. 3.4.13.9). Nucleotide sequence of a gene encoding an OP nerve agent degrading enzyme from Alteromonas haloplanktis was reported in 1997 (22). Biodegradable liposome carriers act as protective environments for metabolizing enzymes that antagonize the toxic effects of various OP agents. This study is focused on the application of a recombinant OP hydrolyzing enzyme, OPAA, whose substrate specificity is amenable to hydrolyzing DFP, sarin and soman.

MATERIALS AND METHODS

Chemicals: DFP was purchased from Sigma (St Louis, MO). DFP solution was prepared by diluting in cold (0-2°C) solution of 0.9 % sodium chloride and was chilled on ice not longer than 2 hours before administration. Atropine sulfate and 2-PAM solutions were prepared daily. All other chemicals used were of the highest purity commercially available.

Enzyme: Purification of OPAA (EC 3.1.8.2) from Alteromonas strain JD6 was reported in 1993 (21) and in 1991 (22).

Encapsulation: Sterically stabilized liposomes (SL) were prepared as previously described (19). Percentage of encapsulation efficiency was calculated from the amount of encapsulated OPAA divided by the amount of OPAA added multiplied by 100.

OPAA Activity Determination in (SL): OPAA activity in liposomes was measured by monitoring the production of fluoride from DFP with a fluoride ion sensitive electrode (Orion Research Inc., Boston, MA) (24). The assay solution for the enzyme fractions contained NaCl (70 mM), KCl (280 mM), Tris (70 mM, pH 7.2), and DFP (3.44 mM). The solution for determining enzyme activity of (SL)* was isotonic: It contained phosphate buffer (10 mM), NaCl (0.144M), $MgCl_2*6H_2O$ (2.0 mM) dextrose (5mM), and DFP (3.44mM), and had the osmolality of 290 mosM. The DFP solution

was kept on ice before using, and the enzyme activity was determined at 25 ± 1°C in a thermostated titration flask (Brinkman Instrument, Inc.). The total volume of the solution was 5.00 ml. Electrode potential was recorded as a function of time on a strip chart recorder (Orion Research Inc, Boston, MA), and the potential values were converted to concentration using the Nernst equation. Results are expressed as the mean +/- 1 standard deviation unless otherwise indicated. One unit of OPAA is defined as that amount of enzyme which hydrolyzed 1μmol of DFP to fluoride and isopropylphosphate per minute.

Animals
Male Balb/C mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighting between 18-20 g were housed in a temperature and light controlled rooms (21 +-2°C, 12-h light/dark, full spectrum lighting cycle) and were furnished with water and 4% fat Rodent Chow (Teklad HSD, Inc., WI) ad libitum. All animal procedures were conducted in accordance with the guidelines by The Guide for the Care and Use of Laboratory Animals (National Academic Press. 1996), credited by AAALAC (American Association for the Assessment and Accreditation of Laboratory Animal Care. International). At the termination of the experiments, surviving animals were euthanized in accordance with the 1986 report of the AVMA Panel of Euthanasia.

In vivo experiments: Male mice received 10-20 units of OPAA intravenously (as the free enzyme or encapsulated within SL*) one hour prior to receiving DFP subcutaneously. No gross toxic effects were apparent in mice receiving encapsulated OPAA, atropine and 2-PAM, either alone or in various combinations. Atropine (10 mg/kg) and 2-PAM (90 mg/kg) were administered intraperitoneally to mice 45 and 15 minutes, respectively, prior to receiving OPAA. Each LD_{50} value was obtained from five or more graded doses of DFP administered to five or more groups of six to eight mice based on 24 h mortality. The LD_{50} values were determined by the method of Litchfield and Wilcoxon (25) as determined by the computer program PHARM/PC Version 4.1 (26). All statistical procedures were performed at the 95% confidence level.

RESULTS AND DISCUSSIONS

Recombinant OPAA was encapsulated into SL by mechanical dispersion method (19) and (17) with minor modifications with an 80 to 90% encapsulation efficiency. Purification of OPAA (EC 3.1.8.2) from Alteromonas strain JD6 has been reported by Cheng, et al. (1993). Sterically stabilized liposome encapsulating OPAA rapidly hydrolyzes DFP to fluoride and diisopropyl phosphate (Figure 1). Enzymatic hydrolysis of DFP with varying amount of (SL)* is shown in Figure 2. Concentration of the (SL)* within each added aliquot was constant and DFP hydrolysis was linear. Formation of fluoride ions was directly proportional to the amount of (SL)* containing OPAA. There was no OPAA activity noted with liposomes containing no enzyme. Increases in amount of (SL)* are equivalent to increases in amount of OPAA in the reaction. DFP hydrolysis catalyzed by the OPAA containing (SL)* was linear over a period of 1 min depending on the concentration of DFP

These sterically stabilized liposome system encapsulated with recombinant OPAA was used in these OP antagonism studies. Prophylactic antidotal protective effects of OPAA (encapsulated and free) with the combination of 2-PAM and/or atropine are summarized in Table 1. (Potency ratio = LD_{50} antagonized / LD_{50} unantagonized). The LD_{50} for the unantagonized DFP is 4.2 mg/kg (control, expt. 1). Atropine alone gave an increase in LD_{50} to 5.7 mg/kg (expt. 2), and 2-PAM alone increase the protection to 7.7 mg/kg (expt. 3). When 2-PAM and atropine were combined, the LD_{50} was increased to 29.3 mg/kg (expt. 4). When free OPAA enzyme without liposomes was administered in a combination with 2-PAM + atropine (expt. 5), the protection of 2-PAM and atropine combination was only slightly enhanced to 33.2 mg/kg. When sterically stabilized liposomes encapsulating OPAA was used in combination with 2-PAM and atropine (expt. 6), a dramatic enhancement was detected: The LD_{50} value was increased to 98.6 mg/kg. The overall protection against DFP intoxication was enhanced from 4.2 mg/kg to 98.6 mg/kg with the 2-PAM, atropine, and (SL)* combination.

The results suggest a potentially new conceptual approach for the rational design of antidotal therapy against chemical poisoning. Presently, there are less than six specific antidotes that exceed the LD_{50} values by five times when protecting against the lethality of chemical poisoning. There is a need for a more successful approach to develop antidotes. Recent developments in molecular biology and biotechnology have made it possible that fast catalytic recombinant enzymes can be rapidly prepared and encapsulated within liposomes with minimal macrophage recognition. By using sterically stabilized long circulating liposomes (SL) the half-life of conventional liposomes of only a few hours can be extended to a half-life of a few days. This permits the development of effective prophylactic and therapeutic agents. These results represent an application of SL as a biodegradable enzyme carrier system for OP hydrolyzing enzyme. OPAA, to degrade OP agents and to antagonize the lethal effects of various OPs including DFP and possibly permit

extrapolations can be made from kinetic data with other substrates such as soman and sarin.

Earlier studies (20) indicated that (SL) encapsulating an other OP degrading enzymes (OPH) provide considerable protection either alone or in a combination with 2-PAM and/or atropine to antagonize the lethal effects of paraoxon. When combined with 2-PAM and atropine, (OPH-SL*) increased the protection over 1000 LD_{50} doses. Preliminary data with paraoxon indicate that the [2-PAM - atropine - (OPH-SL*)] antidotal system is superior to the [2-PAM-atropine-(OPAA-SL*)] system with DFP. However, of greater importance is that (OPAA-SL*) combined with 2-PAM and atropine greatly potentiated the protection against DFP. If our extrapolation from the enzyme kinetic data is valid, protection with (OPAA-SL*) against the nerve gas, sarin, should be superior to DFP, and the protection against soman should be at least the same as against DFP. The enzyme kinetic data (27) indicates that paraoxon has a high affinity to OP hydrolase (OPH), but this enzyme is less effective for DFP, sarin and soman (Table 2). OPAA has higher affinity to DFP, soman and sarin; however, it does not hydrolyze paraoxon and other agricultural OP insecticides effectively. DFP is chemically and kinetically similar to the chemical warfare agents, soman and sarin, therefore it seems to be an appropriate OP model compound in an attempt to predict the protective effects of (OPAA-SL*) against soman and sarin intoxication.

SUMMARY

Antidotal combination for the antagonism of organophosphorus (OP) poisoning in the USA are 2-PAM and atropine. Atropine prevents muscarinic receptor occupation whereas 2-PAM reactivates the OP inhibited acetylcholinesterase, but they don't degrade the OP molecules. In these studies sterically stabilized liposomes (SL) containing recombinant OP hydrolyzing enzyme (OPAA) were employed as a carrier model to antagonize the lethal effects of DFP. DFP serves as a good substrate for OPAA as well as some chemical warfare agents, e.g. soman and sarin. The in vitro properties of this carrier system and the in vivo antidotal protection was studied when it was used in combination with 2-PAM and/or atropine. The hydrolysis of DFP was determined by measuring the increase of fluoride ion concentration using fluoride sensitive electrode. Recombinant OPAA enzyme originated from Alteromonas Strain J6D has a broad substrate specificity for OP compounds, especially for DFP, soman and sarin. The OPAA encapsulated within (SL) could rapidly hydrolyze DFP. The rate of DFP hydrolysis was directly proportional to the amount of (SL)* added to the reaction mixture. (SL) with no enzyme did not hydrolyze DFP. Rate of hydrolysis was first order to fluoride and diisopropylfluorophosphate. The present in vivo studies suggest that the antidotal protection of the classic OP antidotes 2-PAM and/or atropine can be strikingly enhanced when they are used in a combination with the OPAA encapsulated (SL)*. (Supported by grants from NIH, NSF, USAMRDC, NOAA, and NATO)

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KEYWORDS
OPA Anhydrase (OPAA), Sterically Stabilized Liposomes, OP Hydrolase (OPH), Organophorus antagonism. Long Circulating Liposomes

FIGURES AND TABLES

Figure 1. Sterically stabilized liposomes encapsulating OPAA rapidly hydrolyzes DFP.

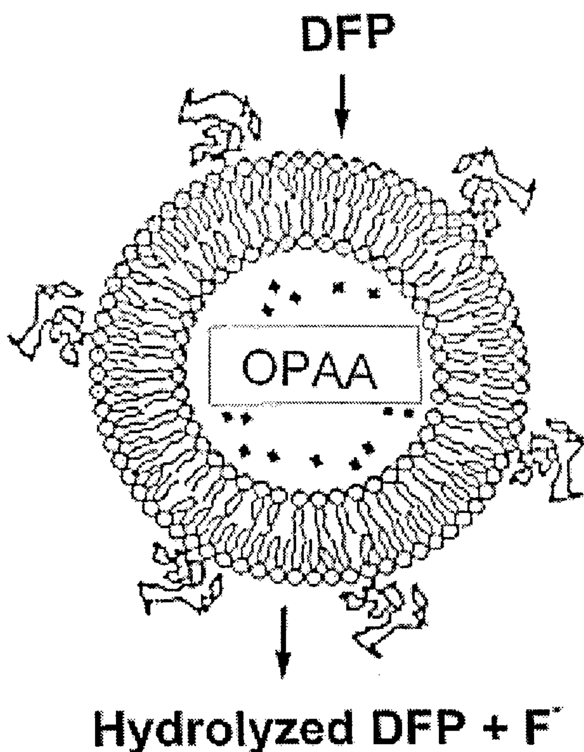

Figure 2. Time course for DFP hydrolysis by OPAA encapsulated within SL.

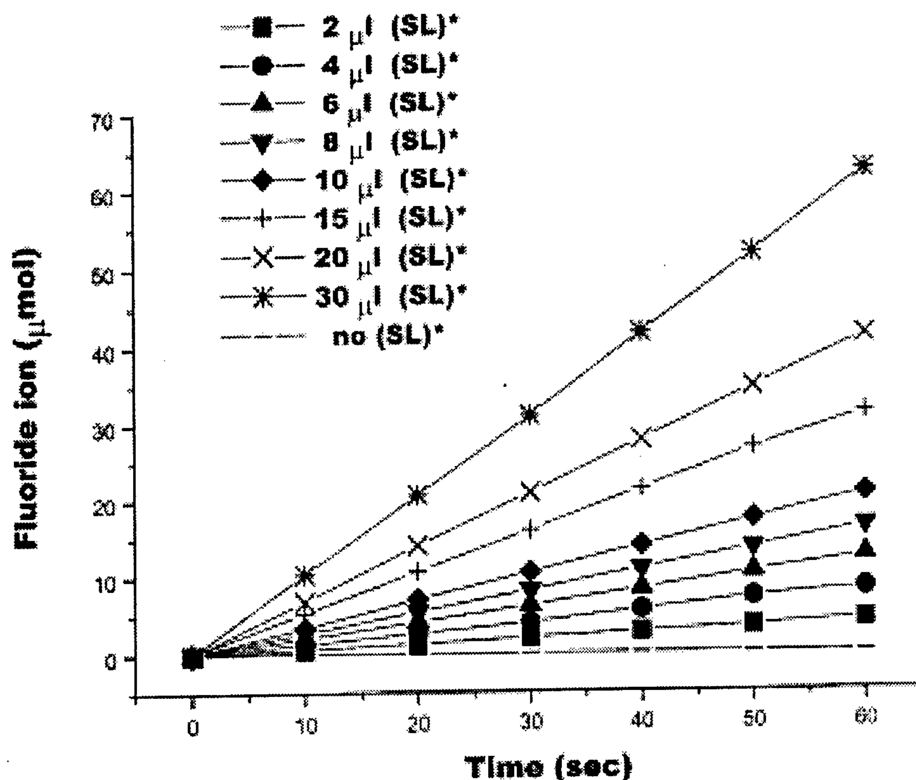

Table 1. DFP Antagonism on Swiss Webster Mice with OPAA

Exp.	Treatments before DFP				LD50 (mg/kg) [a]	Potency Ratio [b]
	OPAA carriers	OPAA (iv)	2-PAM (ip)	Atropine (ip)		
1	Control (no antagonist)	-	-	-	4.2 (3.9-4.6)	1.0
2	(No Enzyme) (no carrier)	-	-	+	5.7 (5.3-6.3)	1.3
3	(No Enzyme) (no carrier)	-	+	-	7.7(7.4-8.1)	1.8
4	(No Enzyme) (no carrier)	-	+	+	29.3 (28.0-30.6)	6.9
5	Free OPAA (no carrier)	+	-	-	7.3 (6.8-7.5)	1.7
6	Free OPAA (no carrier)	+	-	+	7.4 (6.6-8.2)	1.7
7	Free OPAA (no carrier)	+	+	-	10.3(9.2-11.5)	2.4
8	Free OPAA (no carrier)	+	+	+	33.2(31.1-35.4)	7.8
9	Sterically Stabilized Liposomes	+	-	-	9.6(8.9-10.2)	2.3
10	Sterically Stabilized Liposomes	+	-	+	18.9(16.5-19.5)	4.5
11	Sterically Stabilized Liposomes	+	+	-	21.1 (20.0-23.5)	5.0
12	Sterically Stabilized Liposomes	+	+	+	98.6(86.5-104.5)	23.2

a) DFP (3.0-120 mg/kg) was delivered subcutaneously to mice. Atropine sulfate (10 mg/kg) and 2-PAM-CI (90mg/kg) was given intraperitoneally, 45 and 15 minutes respectively, prior to receiving DFP. (SL)* (20-30 units/mice) was given intravenously though tail vein one hour prior to receiving DFP.

b) Potency Ratio = $\frac{\text{LD50 of DFP with antagonists}}{\text{LD50 of DFP without antagonists}}$

Table 2. Comparison of kinetic behavior of some OP compounds with OPH and OPAA

Substrate	Paraoxon	DFP	Soman	Sarin
Enzyme	kcat			
OPH	41400	410.00	9.6	80
OPAA	0.43	76.8	60.9	281.5

87. DELAYED TOXIC EFFECTS: EMERGING NEW CHALLENGES TO CHEMICAL BIOLOGICAL RISK ANALYSIS AND MEDICAL TREATMENT STRATEGIES

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ABSTRACT

To date, predictive toxicology of delayed effects associated with exposure to chemical and biological risk factors remains mostly in the realm of what is referred to as the "expert judgement." Noteworthy accomplishments during the past five decades, particularly in the areas of chemical carcinogenesis, neuro- and developmental toxicology have contributed to risk management decisions on chronic effects grounded on improved scientific principles. Despite progress in these areas, our understanding of chronic health and environmental consequences of exposure to toxic agents is limited, and shrouded with uncertainties. Research priorities in the crucial areas of long-term toxic effects and hazard assessment approaches are not in tandem with risk management decision process involving chemical and biological medical treatment and mitigation. New challenges such as terrorist threats and extended commitments with peace-keeping operations worldwide requires a coordinated assessment of the tools currently available for delayed toxic effects resulting from exposure to chemical and biological agents. This paper will present a conceptual chemical class-specific approach to delayed toxic effects and draw example data sets from organophosphate compounds in civilian and military uses.

INTRODUCTION

The scope of delayed toxic effects of chemical and biological toxic agents of anthropogenic origin is an area of increasing focus both for the scientific community and government agencies involved in health and environmental regulation. A new dimension covering national security was introduced to the delayed toxicity analysis, which until recently was perceived as a public health and environmental problem. Although literature is replete with information on the acute and sub-chronic toxicities of chemical agents, information is scant from published human and experimental animal studies on the delayed effects of military-unique chemicals (SIPRI, 1975). Recently, the U.S. Army requested the National Research Council to perform a review and suggest methods to develop sub-chronic toxicity criteria for chemical weapon agents and degraded constituents (NRC, 2000).

Chemical-induced residual lesions, caused by acute or sub-acute exposures may result in essentially irreversible clinical manifestations (Munro, 1994). There is considerable literature both from human and experimental animal studies on the chemical-induced carcinogenesis and developmental toxicities with dose-response relationships spanning the life span following a period of exposure (Watson et al. 1989; Rao et al. 1989). Additionally, in the "real world", simultaneous or sequential exposure to low levels of multiple chemicals and biological risk factors presents additional risks due to interactive effects and leading to potentiation of delayed adverse impact.

A key distinction is that delayed toxic response is not the same as chronic adverse effects. For instance, published literature on Organophosphorus (OP) pesticides and chemical weapons (CW) agents has revealed that the lesions associated with delayed effects are dissimilar to those attributed to chronic poisoning (SIPRI, 1975). In contrast to chronic exposure-which involves continuous or intermittent exposure to low levels of a toxic agent-delayed toxicity may result from a single dose or a brief exposure producing an irreversible effect. As a result, dose, duration and exposure considerations for delayed toxicity are not comparable to those for chronic exposure.

Thus, delayed toxicity evaluation and hazard assessments in cases involving exposure to low-levels of OP-based chemical toxicants is a major challenge to design and development of rationale medical treatment strategies. As dual-use agents, OP compounds are ideal both for delayed toxicity analysis and to assess potentials for interactive effects. A collective hazard analysis of OP-induced delayed effects was considered necessary because of overlapping military and civilian applications of this class of compounds. Environmental releases due to widespread civilian uses of OP compounds and releases in the military operational environment may introduce levels hazardous to public health and general environment.

Over the past 15 years we have been involved in the analysis of interactive effects of large classes of chemical carcinogens including OP chemicals with a goal to develop better tools for hazard and risk analysis (Rao, 1991a, 199b, 1992). We have compiled large databases on the binary combination effects of chemicals and chemical groups on delayed effects such as carcinogenesis (Arcos et al. 1988, Rao et al. 1989), developed mechanistic-based tools for toxicity and hazard assessment of chemical mixtures (Rao and Unger, 1995; Rao 1991a), expert systems to elucidate

patterns in the interactive effects of chemical mixtures and delayed toxic effects such as tumor promotion (Polanksy et al. 1989; Rao et al. 1989).

This paper will outline: (a) approach in the analysis of select data sets from our studies involving a combination both of delayed toxic effects and opportunities for interactive effects, (b) development of a mechanistic-based toxicity parameters for delayed effects for OP compounds, (c) derive preliminary environmental concentrations protective of delayed toxicity endpoints, and (d) draw general conclusion on the role of chemical-class specific decision points on medical management issues.

MATERIALS & METHODS

Conceptual Premise: Published studies have reported specific cellular or molecular lesions as the underlying mechanism for delayed toxicity demonstrated by anthropogenic chemical agents. For instance, OP-induced cellular lesions associated with delayed neurotoxicity involves, (a) inhibition of the neuropathy target esterase (NTE), a membrane-bound protein with high esterase catalytic activity, and (b) progressive aging of the inhibited NTE less amenable to catalytic reactivation (Johnson, 1990; Jokanovic and Hohnson, 1993).

OP induced delayed polyneuropathy (OPIPN) is linked to the irreversible inhibition of NTE (Johnson, 1993). These effects are unrelated to the wide array of adverse acute toxicities associated with the inhibition of acetylcholinesterase. The potentials for delayed effects associated with exposure to low levels of OP are unclear, although limited epidemiological and controlled clinical trial studies have revealed transient neurological and neurobehavioral effects (Ray, 1998).

The striking similarity in the acute and delayed toxicity profiles of OP chemicals is attributed to the overlapping chemical structural and biochemical functional characteristics of this class of compounds. Notably, the toxicity profiles, including NTE-dependent delayed effects of divergent categories of OP chemicals such as insecticides, pesticides and rodenticides, and an entire class of CW agents is mediated via common molecular mechanism(s) of action. A central requirement is the need to develop toxicity parameters for hazard assessment.

Selection of Data Sets: Published data on OP chemicals from the pesticide groups and CW agents were compiled for a comparative analysis of the dose-duration-response relationships. OP compounds are a divergent category of chemicals with closely similar chemical structural and molecular mechanisms producing delayed toxic effects both in humans and experimental animals. Interestingly, the environmental transport and fate characteristics of OP compounds are similar. Structure-activity relationship studies have revealed interactive effects leading to potentiation of delayed toxic effects (Jokanovic and Johnson, 1993; Lotti and Moretto, 1999). Hence occupational or environmental exposures to OP compounds of divergent uses may posed risks for delayed long-term effects. .

Our comprehensive literature survey and analysis covered short-term and long-term studies on representative categories of OP pesticides and CW agents. A database on the reported short and delayed adverse effect together with dose and duration of exposure was compiled to prepare matrix of dose-duration-response relationships of various categories of OP compounds. Data both from epidemiological studies and animal experimentation were compiled and consolidated. Data on the analytical detection limits in the environmental media were collected for select OP compounds.

Dichlorvos (DV), a OP pesticide, was selected for a comparative dose-response analysis with OP CW agents because (a) DV is a halogenated OP compound like some OP CW agents, (b) have comparable physicochemical and environmental properties, (c) causes NTE-mediated delayed neurotoxicity (Sarin and Gill, 2000), and (d) DV has a well-defined reference dose for hazard and risk assessment (IRIS).

Relative Hazard Assessment: Short- and delayed toxicity parameters including macromolecular effects were identified, sorted and compiled for inclusion in the database. The database comprised of: No Observed adverse effects levels (NOAEL), Low observed adverse effects level (LOAEL), maximum tolerated dose (MTD), US EPA suggested reference dose (RfD) for risk analysis, and lethal doses (LD_{50}). Effective dose (ED50) reported for biochemical, toxicological and physiological endpoints. Time course for development of toxicity was obtained either directly from the published studies or based on the experimental design (acute, sub-chronic, and chronic). By design, epidemiologic studies report sub-chronic and delayed effects, with limited information on the dose-duration and exposure relationships.

Standard exposure and risk analysis algorithms were used to calculate an estimated reference dose and environmental concentrations for OP CW agents (EPA, 1989). Based on an assumption involving exposure via ingestion and dermal contact and varying exposure duration, soil concentrations were estimated that represent intake doses for delayed effects. Note that these values may change markedly if exposure assessment is performed for

combined exposures involving OP compounds in civilian uses. Legend under Table 1 lists the exposure factors used in the algorithms.

RESULTS AND DISCUSSION

Figure 1 reveals a considerable convergence in the post exposure developmental profile for toxic effects for the representative OP pesticides and CW compounds. The matrix for the toxicity profile indicated. (a) casual relationships between macromolecular effects and immediate and mid-term toxicities in experimental animal studies, (b) weak casual relationships for delayed toxicity both in human and experimental animal data. (c) existence of possible mechanistic linkages between macromolecular effects and toxicity, (d) irreversible nature of adverse effects, particularly with delayed responses, and (e) delayed effects manifested as a syndrome involving multiple physiological systems.

Published analyses on the elucidation of toxicity development of OP CW compounds were largely based on OP pesticide and derivatives. Likewise, delayed toxicity data such as carcinogenicity, mutagenicity and developmental toxicity are almost solely on OP pesticides and derivatives. Nevertheless, structure-activity relationship considerations and similarity in molecular mechanism of action form the basis for a meaningful comparison of delayed toxicity development between classes of OP compounds.

Figure 2 illustrates the relative magnitude of dose-response relationships between classes of OP compounds. Evidently, dose-response relationships between the classes of compounds differ by several orders of magnitude for comparable toxicity parameters. For instance, the reported estimated human lethal dose (LD_{50}) of 0.01 mg/kg for OP CW compounds was less than the NOAEL dose of 0.05 mg/kg-day for DV in experimental animals. All published sub-lethal and lethal doses of VX in the published literature were well below the 0.05 mg/kg-day NOAEL dose of DV. In contrast, published ED_{50} - LD_{50} range for halogenated OP CW compounds such as Sarin (0.04-0.16 mg/kg) and Soman (0.004-0.13 mg/kg) were in the NOAEL-LOAEL-MTD range (0.05-16.3 mg/kg) of DV in experimental animals.

In the absence of a more scientifically defensible criteria for delayed toxic effects, RfD may be the nearest surrogate toxicity parameter to assess the relative delayed impact of very low concentrations of OP compounds on human health. Given the general lacunae of sub-chronic endpoints for OP CW agents, an alternative approach would involve extrapolation of the toxicology and medical literature of OP based compounds for delayed toxicity among populations potentially exposed to these classes of compounds either during military operations or at low levels in the general environment. A linear extrapolation of the relative differences in the scale and magnitude of threshold doses for adverse effects provided an estimate of NOAEL/LOAEL doses associated with delayed effects. However, this approach applies only for assessment of impact from exposure to individual compounds but not mixtures of OP compounds of differing origin and uses.

EPA has estimated a reference dose (RfD) of 0.0005 mg/kg-day for DV based on the study reporting decreased rat brain cholinesterase activity; NOAEL of 0.05 mg/m3 based on the estimated inhalation dose (Blair et al. 1976, AMVAC, 1990). Based on a comprehensive review of the database, the overall in the RfD was rated as medium (EPA, 1993). Published LOAEL (oral) dose of 0.1 mg/kg-day for DV is 200 times the RfD (0.0005 mg/kg-day). Derivation of RfD value was based on the strengths and weakness in the principal study identified for this purpose (Blair et al 1976; AMVAC 1990).

Adopting a similar approach a tentative RfD of 0.00002 mg/kg-day for OP CW agents was estimated that is equivalent to 200 times the LOAEL (0.004 mg/kg) adjusted for averaged daily exposure. Based on a review of over a dozen studies for sub-chronic toxic effects, a recent report by Sirocco et al. (1990) was used to derive a tentative LOAEL. In this study, researchers reported neurobehavioral effects in male Wistar rats to single intra-peritoneal subtoxic doses of Soman even at the lowest single dose of 0.004 mg/kg. For the purposes of deriving a tentative RfD this value was treated as a LOAEL dose. Uncertainty factors were higher due to lack of doses below the LOAEL. Using a linear extrapolation approach, RfD values of 0.000008 mg/kg-day and 0.000004 mg/kg-day were derived that are equivalent of 500 and 1000 times the LOAEL doses are respectively.

Using standard risk analysis tools, hazard analysis was performed for combinations of intake doses in the LOAEL and RfD dose range. Table 1 is a summary of the estimated soil concentrations for different intake rates and exposure duration. Standard EPA-recommended algorithms were used to derive estimated soil concentrations for the ingestion (oral route) and topical (dermal) routes of exposure. Evidently, soil concentrations (mg/kg) are inversely related to estimated intake doses (mg/kg-day) and exposure duration. Thus, screening-level soil concentration for delayed effects was higher when exposure duration and intake rates were lower and decreased as exposure duration and/or intake rate increased.

Differences in the route of exposure on estimated soil concentration for delayed effects were significant with oral route being the most sensitive route (Table 1). Ratio of dermal/oral soil concentrations corresponding to dermal

exposure was anywhere from 1000 to 8000 with the highest ratio at the maximal exposure duration of 24 years. Strikingly, ratio of dermal/oral estimated soil concentrations were similar for all other exposure durations included in the assessment. Higher soil concentrations corresponding to dermal route reflects the uncertainties associated with this exposure pathway and assumptions used in the derivation of intake doses (Table 1, footnote).

With a growing threat of terrorism and release of chemical residues to general environment from chemical demilitarization programs, there is an increasing awareness of the far reaching environmental and legal liabilities among governmental agencies and the private sector.

The approach outlined in this paper may form the basis for a more formal assessment of the literature on delayed toxicity of OP compounds based on structure activity relationships and dose-duration-response relationships. This approach may help devise and develop medical and public health intervention strategies for OP CW compounds and breakdown products in the environment. The conceptual premise for the derivation of an RfD for oral route was based on the comprehensive database on OP CW from human and animal studies and follows the standard linearized assumption on the LOAEL and RfD dose-relationships.

However, there are several shortcomings with the approach outline here: (a) Data gaps on dose-response relationships of OP CW compound and use of a simplified assumption of linearity in the extrapolation of LOAEL doses to derive a tentative reference dose, (b) Exposure assumptions are based on exposure to single chemicals and not chemical mixtures, (c) LOAEL dose not constitute delayed toxic effect, (d) It is unclear as to how the outcome this analysis would help devise and develop the nature of medical intervention measures.

Additional studies on the available data together with a focus to consolidate the available data on OP compounds of all uses is necessary in order to develop framework for a more structured analysis.

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FIGURES AND TABLES

Figure 1: Post Exposure Duration in the Development of OP CW Compounds-Induced Delayed Toxicity*

Reversible (Except DNA Effects) Immediate Toxic Symptoms Medical Intervention Most Effective Better Causal Relationship	Partly Irreversible Gradual development of symptoms Nongenotoxic Limited causal relationship	Irreversible Delayed Toxicity Genotoxic Pronounced epigenetic effects Limited causal relationship Medical intervention minimally effective	Irreversible Delayed Toxicity Poor causal relationship Complex syndromes Medical intervention minimally effective
Inhibition of Chlonesterase (Acylaton)–Acute toxicity Alkylation of DNA–Initiation of Delayed Toxicity Phosphorylation of biogenic amines	Hepatotoxic effects Hematologic (CVS) Effects Central Nervous System (CNS) Effects	Mutagenicity Carcinogenecity Developmental Effects Immunotoxicity	Medical syndromes (paralysis, impotence) Polyneupathy (general) OPIDP Cognitive Effects (neuro-behavioral)

Immediate Toxicity	*Intermediate Effects*	*Delayed Effects*
(1-30 days)	*(30-300 days)*	*(> 1 year)*

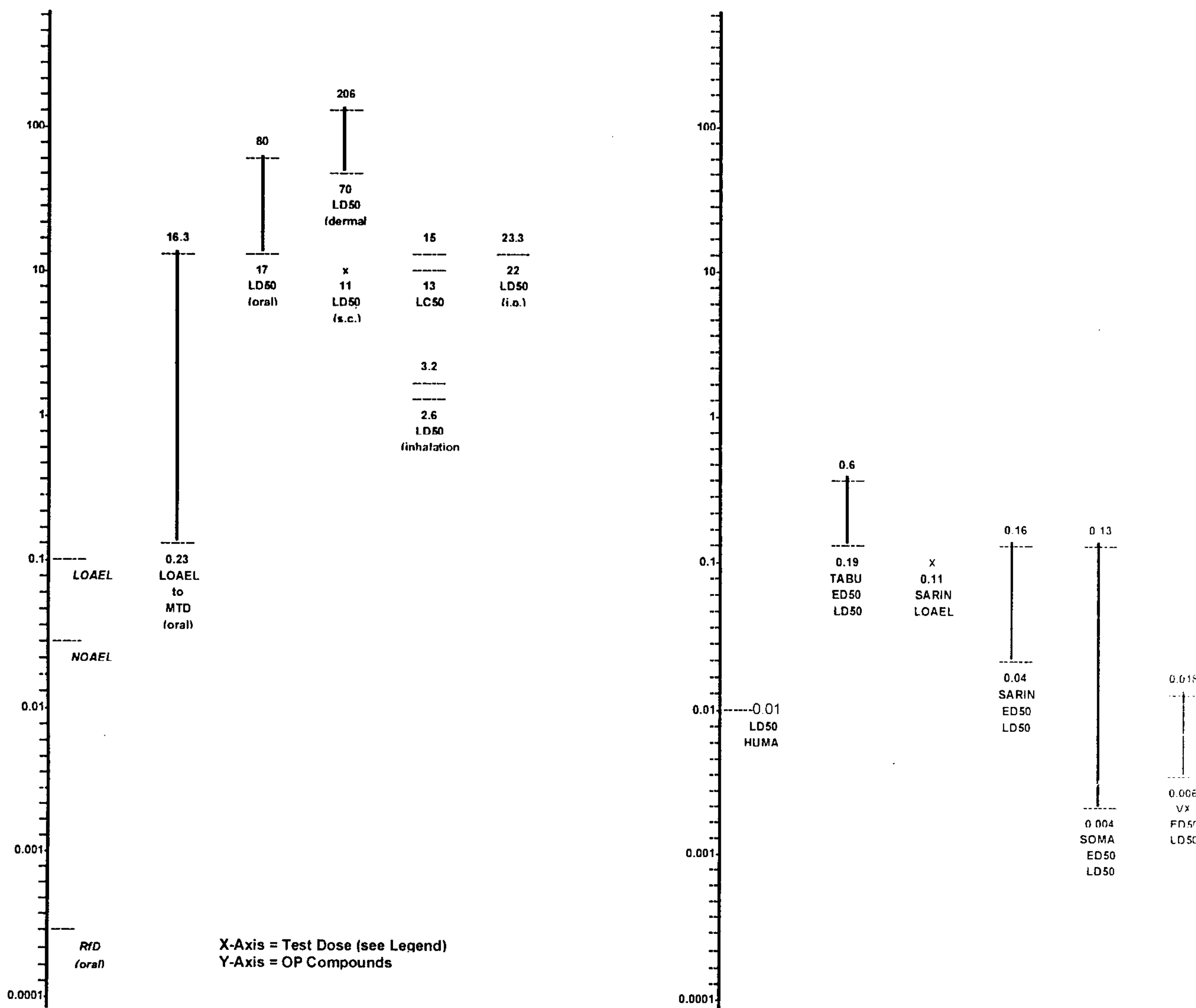

Figure 2: Comparative Dose-Response Analysis for Short and Delayed Toxic Effects of OP Pesticides and CW Agents. Dose Response Pattern from Studies on Experimental Animals

Legend: RfD = Reference Dose; NOAEL = No Observed Adverse Effects Level; LOAEL = Lowest Observed Adverse Effects Level; LD_{50} = Lethal Dose for 50% test population;

ED50 = Effective Dose for responses in 50% population; LC50 = Lethal Concentration (ambient levels) for response in 50% population; MTD = Maximum Tolerated Dose;

i.p. = Intraparetoneal route; s.c. = subcutaneous route.

Units: RfD, NOAEL, LOAEL and MTD = mg/kg-day; LD_{50} and ED_{50} = mg/kg; LC50 = mg/m3 (ambient)

Table 1. Intake Dose and Environmental Concentrations in the Health Hazard Assessment for Delayed Toxicities of OP-Based CW Compounds

INTAKE DOSE (mg/kg-day)	*ENVIRONMENTAL CONCENTRATIONS (mg/kg)*							
	Ingestion Exposure Duration (years)				*Ingestion Exposure Duration (years)*			
	24	5	1	0.5	24	5	1	0.5
0.0005	182	876	4,380	8,760	1.80E+06	9.00E+06	4.50E+07	9.00E+07
0.00004	15	70	350	701	15,051	72,247	3.60E+06	7.20E+06
0.00002	7	35	175	350	7,525	36,123	1.80E+06	3.60E+06
0.000008	**3**	**14**	**70**	**140**	**3,010**	**14,449**	**72,247**	**1.40E+06**
8E-07	0	0	7	14	30	1,444	7,224	14,449
8.00E-10	0.0003	0.001	0.007	0.014	0	0	0	0

Assumptions in the ingestion pathway: Ingestion rate = 200 mg of soil per event; Exposure frequency = 50 days per year;
Exposure duration = 24-0.5 years; Body weight = 70 kg; Averaging factor = 8,760.
Assumptions in the dermal exposure pathway: Skin surface area available for contact = 1.94 cm^2/event; Soil to skin adherence factor = 0.1 (mg/m^3);
Absorption factor = 100 percent; Exposure frequency = 350 days per year;
Exposure duration = 24 to 0.5 years; Body weight = 70 kg; and Averaging factor 8,760.
Exposure assumptions in the intake equations are based on conservative values used (EPA, 1989).

89. PRIOTRITIZING POTENTIAL BIOLOGICAL TERRORISM AGENTS FOR PUBLIC HEALTH PREPAREDNESS IN THE UNITED STATES: OVERVIEW OF EVALUATION PROCESS AND IDENTIFIED AGENTS

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ABSTRACT

Many biological agents have the capacity to cause disease and potentially be used to threaten civilian populations. From a public health standpoint, however, biological agents must be evaluated and prioritized in order to assure appropriate allocation of the limited funding and resources that are often found within public health systems. This paper describes a process to help identify and prioritize potential biological terrorism agents for public health preparedness activities including: 1) public health impact of agent release, 2) delivery potential to large populations, 3) public perception of an agent, and 4) special public health preparedness requirements for detection and response to an agent release. The identified priority biological agents and public health activities targeted for these agents are also discussed.

INTRODUCTION

Although the use of biological and chemical agents in military conflicts has been a concern of military communities for many years, several recent events have increased the awareness regarding the potential use of these weapons by terrorists against civilian populations. The release of sarin gas in Tokyo subways by the Aum Shinrikyo cult resulted in several deaths and many casualties in the exposed commuters.[1] Other attempts to use biological agents by this group were discovered during further investigations of the subway attacks. In 1990 this group tried to spray botulinum toxin into several government buildings and in 1993 they attempted to release anthrax spores from their Tokyo office building laboratory.[2] Although no known illnesses resulted from these attempts to use biological agents as weapons, the exploration of the use of these agents by this and other extremist organizations caused great concern regarding the potential vulnerabilities of civilians to such weapons.

Because of this concern, the United States public health community began to address preparedness needs for detecting and responding to a release of a bioterrorism agent within the U.S. In order to guide preparedness activities and allow appropriate allocation of funding, a process was developed to help evaluate and prioritize biological agents that posed the greatest threat to public health.

MATERIALS AND METHODS

The biological agents evaluated were selected from the following published lists of previously identified threat agents: 1) the Select Agent Rule, 2) the Australian Group List of Biological Agents for Export Control, 3) the unclassified military list of biological warfare agents, 4) the Biological Weapons Convention list, and 5) the World Health Organization Biological Weapons list.[3-7]

Selected public health, infectious diseases, military, and national security experts were asked to suggest possible criteria that could be used to evaluate potential threat agents. Four general areas of consideration were used to evaluate biological agents: 1) the public health impact based on morbidity and mortality, 2) the delivery potential to large populations based on environmental stability of the agent, ability to mass produce and distribute a virulent agent, and the potential for person-to-person spread, 3) heightened public perception or fear of an agent and potential for civil disruption, and 4) special preparedness needs based on therapeutic stockpile requirements, enhanced surveillance, diagnostic, and/or education needs. Rating schemes were then developed for each of these general categories for agent evaluations. Potential agents were evaluated according to this rating scheme and placed in one of three priority categories.

Potential biological terrorism agents were given a higher rating (++) for morbidity if clinical disease required hospitalization for treatment (including supportive care), and a lower rating (+) if outpatient treatment was possible for most cases. Agents with an expected mortality of $\geq$50% were rated higher (+++) than agents with lower expected mortalities (21-49% = ++, and <21% = +). Agents received + to +++ for

dissemination potential based on their environmental stability after release (+), and their ability to be produced (+) and distributed (+) as a virulent agent in quantities that could effect large populations. Transmissible agents were also rated (+ to +++) based on their infectiousness (infectious by contact, respiratory route, or both). Public fear associated with an agent and the potential mass civil disruptions that may be associated with even a few cases of disease were also considered (+ to +++). Finally, agents were ranked based on any special public health preparedness that was required including: 1) stockpiling of therapeutics (+), enhanced surveillance and education (+), and improved laboratory diagnostics (+).

Placement into a final priority category was based on public health judgment of the ratings received in each category and a public health judgment of the overall relationships between the categories. For example, if an agent received a high rating in the public health impact category because of a high morbidity and/or mortality but had a low dissemination potential or required fewer special preparedness activities it was placed in a lower priority category as it either posed a lesser public health threat as a bioterrorism agent or did not require enhanced public health preparedness. And although disease caused by an agent may have a low mortality rate and be treatable as an outpatient, it was placed in a higher priority category if it could be disseminated to affect a large number of people or had special preparedness needs.

RESULTS AND DISCUSSION

Rankings of several potential bioterrorism agents are shown in Table 1. Once ranked, agents were placed in one of three priority categories [Table 2]. Agents in Category A were considered to have the highest public health impact potential and were established as priority agents for all preparedness activities. Public health preparedness efforts for agents in Category B focused on specifically identified deficiencies for each agent such as improved awareness, surveillance, or diagnostic capabilities while Category C agents were addressed using previously established public health surveillance and diagnostic systems.

SUMMARY

Although many biological agents can be used to cause illness, there are only a few that can truly threaten civilian populations on a large scale. If released upon a civilian population, these agents would pose the most significant challenge for public health and medical responses. The above criteria for ranking potential biological agents of greatest public health concern was developed and used by the Centers for Disease Control and Prevention to determine priority biological threat agents for national public health preparedness efforts for bioterrorism. Having a defined method for evaluating biological threat agents allows for a more objective evaluation of newly emerging potential threat agents, as well as continued re-evaluation of established threat agents. Using this prioritization method can help focus public health activities related to bioterrorism detection and response and assist with the allocation of limited public health resources.

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KEYWORDS

Biological terrorism, biological agent ranking, public health preparedness

FIGURES AND TABLES

Table 1. Criteria and Ranking for Several Potential Biological Terrorism Agents

	Public Health Impact		**Dissemination Potential**		Special	
Disease	Morbidity	Mortality	Stable/Produce/Distribute	Person Spread	**Preparedness**	Pe
Smallpox	+	++	++	+++	+++	
Inhalational anthrax	++	+++	+++	-	+++	
Pneumonic plague	++	+++	++	++	+++	
Botulism	++	+++	++	-	+++	
Tularemia	++	++	++	-	+++	
VHF*	++	+++	+	+	+++	
Glanders	++	+++	++	-	++	
VE†	++	+	++	-	++	
Q fever	+	+	++	-	++	
Brucellosis	+	+	++	-	++	
Toxins‡	++	++	+	-	++	
HPS§	++	++	+	++	none	
Nipah virus encephalitis	++	++	-	-	+	

* Viral hemorrhagic fevers caused by filoviruses (Ebola, Marburg) and arenaviruses (Lassa, Machupo,)
† Viral encephalitis caused by alphaviruses (Venezuelan, Eastern, and Western equine encephalomyelitis viruses)
‡ Biological toxins (e.g. ricin, Staphylococcal enterotoxin B)
§ Hantavirus pulmonary syndrome

Table 2. Biological Threat Agents and Categories for Public Health Preparedness

Category A		
Biological Agent(s)	Disease	Inhalational anthrax
Variola virus	Smallpox	
Bacillus anthracis		
Yersinia pestis	Pneumonic plague	
Clostridium botulinum toxins	Botulism	
Francisella tularensis	Tularemia	
Filo and Arenaviruses*	Viral hemorrhagic fevers	
Category B		
Coxiella burnetii	Q fever	
Brucella spp.	Brucellosis	
Burkholderia mallei	Glanders	
Burkholderia pseudomallei	Melioidosis	
Alphaviruses†	Encephalitis	
Rickettsia prowazekii	Typhus	
Toxins‡	Toxic syndromes	
Chlamydia psittaci	Psittacosis	
Food and water safety threat agents§	Diarrheal disease	
Category C		
Emerging infectious disease agents (e.g. Hantavirus, Nipah virus)		

* Filo (Marburg, Ebola) and Arenaviruses (e.g. Lassa, Machupo)
† Venezuelan, eastern, and western equine encephalomyelitis viruses
‡ Biological toxins (e.g. ricin, Staphylococcal enterotoxin B)
§ Food agents (e.g. *Salmonella* spp., *Shigella dysenteriae*)
Water agents (e.g. *Vibrio cholera*, *Cryptosporidium parvum*)

90. RECOMBINANT CHOLINESTERASES: DEVELOPING AN IDEAL BIOSCAVENGER FOR PROTECTION AGAINST ORGANOPHOSPHATE NERVE AGENTS

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INTRODUCTION

Cholinesterases (ChEs) were shown to be effective prophylactic agents in preventing organophosphate (OP) toxicity in mice and rhesus monkeys (1,2). Although the use of enzyme as a single pretreatment drug for OP toxicity was sufficient to provide complete protection, a relatively large (stoichiometric) amount of enzyme was required to neutralize OP *in vivo*. Significant improvements in OP/enzyme stoichiometry were achieved *in vitro* as well as *in vivo* by combining enzyme pretreatment with oxime reactivation, so that the aging of OP-inhibited FBS AChE was minimized and the catalytic activity of AChE was rapidly and continuously restored (3).

Several recent studies have demonstrated that mutations of the key residues in the active-site gorge of ChEs can have a dramatic effect on the catalytic properties of the enzyme. Using this technique it is possible to obtain mutant enzymes which possess an increased affinity for OPs (4), or are more easily reactivated by oximes (5), and/or possess a reduced rate of aging (6,7). In an effort to improve the bioscavenging performance of ChEs by site-directed mutagenesis, we developed mutants of mouse (Mo) AChE, F295L, F297I, and E202Q AChE. The effect of these mutations on the rate of inhibition, reactivation by HI-6, and aging of Mo AChE inhibited by the two potent stereoisomers of soman were determined. The *in vitro* detoxification of soman and sarin by wild-type, F295L, F297I, and E202Q AChE in the presence of 2 mM HI-6, were also studied.

ChEs are highly glycosylated proteins, with up to 24% of their molecular weight consisting of carbohydrates, which are present primarily as asparagine-linked side chains (8, The successful use of plasma-derived ChEs as OP bioscavengers stems from their relatively long residence time in circulation. To evaluate the possible use of recombinant (r) ChEs as bioscavengers *in vivo*, we compared the mean residence time (MRT) in circulation of five tissue-derived and two rChEs i.v. injected in mice with their oligosaccharide profiles. The results detail the carbohydrate composition and oligosaccharide profiles of recombinant ChEs. The comparative pharmacokinetic study allowed us to examine the possible relationship between protein size, monosaccharide composition, fraction of acidic oligosaccharides and circulatory stability of ChEs from diverse sources.

MATERIALS AND METHODS

Wild-type and mutants of Mo AChE were expressed, purified and characterized with respect to catalytic parameters as described (9). Soman and sarin were obtained from the Chemical Research, Development and Engineering Center (Aberdeen Proving Ground, MD). The two P (-)-epimers of soman were obtained as described (10). Concentrations of soman solutions were determined by titration of the solution with a known amount of fetal bovine serum (FBS) AChE and measurement of residual activity (1 nmol of FBS AChE is equivalent to 400 units). The oxime, HI-6, was obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (Silver Spring, MD). The monomeric (m) and tetrameric (t) forms of FBS AChE were resolved by gel permeation chromatography of purified FBS AChE on Biogel A 1.5 m column (1.5 x 170 cm) equilibrated with 50 mM sodium phosphate, pH 8.0. Dr. Patrick Masson and Dr. Israel Silman provided purified BChE from human serum (HuS) and AChE from Torpedo californica. Dr. Oksana Lockridge and Dr. Palmer Taylor provided purified recombinant human (rHu) BChE and recombinant mouse (rMo) AChE, respectively.

The inhibition, reactivation, aging, and detoxification studies with rMo AChEs were performed as described (11). The glycan analysis and pharmacokinetic studies were conducted as reported previously (12).

RESULTS AND DISCUSSION

Single site substitutions of glutamate E202 (*199*) located next to the active-site serine S203 (200) with glutamine, or phenylalanines at positions 295 (*288*) and 297 (*290*) located in the acyl pocket of Mo AChE with leucine and isoleucine, respectively, generated mutant enzymes with altered inhibition and reactivation properties. To examine the effect of these mutations on the stereoselectivity of AChE, we compared the bimolecular rate constants for the inhibition of wild-type, F295L, F297I, and E202Q AChE by the two P(S)-diastereomers of soman (Table 1). Racemic soman is a mixture of four stereoisomers of which only the two P(S)-diastereomers are potent inhibitors of AChE (13,14). Although the reactivity of F295L AChE toward the two diastereomers of soman was similar to that

of wild-type AChE, mutations of phenylalanine at position 297 and glutamic acid at position 202 caused a 5- to 10-fold decrease in the bimolecular rate constants for inhibition by these diastereomers. A 2-fold difference in the bimolecular rate constant of wild-type AChE for the two P(S)-diastereomers was observed compared to the 3- and 4.5-fold differences observed for F295L and E202Q AChE, respectively. Previous inhibition studies with stereoisomers of soman also showed that the P(S)C(S)-soman was a slightly more potent inhibitor of ChEs compared to P(S)C(R)-soman (14,15). However, mutations of the two residues in the acyl pocket did not affect the reactivity of mutant enzymes toward sarin.

The influence of these mutations on 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxyaminopyridinium)-dimethyl ether hydrochloride (HI-6) induced reactivation of wild-type and mutant AChEs inhibited by the two P(S)-diastereomers of soman were also studied. For AChE inhibited with either of the two P(S)-diastereomers of soman, the second order reactivation rate constant for F297I AChE was essentially the same as that for wild-type AChE (Table 1). However, a 4- to 6-fold increase and an 8- to 10-fold decrease in the rate of reactivation were observed for F295L and E202Q AChE, respectively. For all enzymes, P(S)C(R)-inhibited-AChE reactivated at a 7- to 10-fold slower rate as compared to P(S)C(S)-inhibited-AChE. Similar effects were demonstrated for the reactivation of racemic 7-(methylethoxyphosphinyloxy)-methyquinolinium iodide (MEPQ)-inhibited E202Q and F295L Mo AChE by 2-PAM and HI-6 (5). For the F295L Mo AChE, the enhancement was observed with half of the racemic MEPQ-inhibited enzyme (5), and the second half appeared to reactivate at an extremely slow rate. For E202Q Mo AChE, the mutation affects the rate of reactivation as well as the extent of reactivation especially with P(S)C(R)-soman-AChE. These results are consistent with molecular modeling studies which showed that steric hindrance between the methyl group at the chiral carbon of P(S)C(R)-soman and H440 can reduce the efficiency of nucleophilic reactivation of P(S)C(R)-soman-AChE compared to P(S)C(S)-soman-AChE (16). The results are also consistent with *in vitro* studies with human, electric eel, and Plaice AChE, which demonstrated that reactivation of P(S)C(S)-soman-AChE by HI-6 was more effective than the reactivation of P(S)C(R)-soman-AChE (14,15).

A reaction that counteracts reactivation of soman-inhibited AChE is aging. We examined the rate of aging of wild-type and mutant AChEs inhibited by the two P(-)-diastereomers of soman by their incubation at pH 8.0, and subsequent measurement of the extent of reactivation by 2 mM HI-6. No differences in the rate constants for the aging of wild-type AChE inhibited with P(S)C(S)- and P(S)C(R)-soman were observed. These results are consistent with previous observations made with human, eel, and bovine erythrocyte AChE, which showed that the rate of aging was practically independent of the configuration at the _-carbon atom of the pinacolyl moiety of soman (14,17). Replacement of phenylalanines in the acyl pocket did not have any significant effect on the rate of aging at pH 8.0. However, for E202Q AChE, at pH 8.0 and above, the rate of aging was so slow that it could not be measured under present experimental conditions (Table 1).

Aging has proven to be the major barrier to achieving oxime reactivation of AChE inhibited by the more potent OPs (3). Recombinant enzymes without this liability would confer a superior characteristic in the development of catalytic scavengers of OPs. To test this possibility, we carried out *in vitro* detoxification of soman and sarin by wild-type F295L, F297I, and E202Q AChE in the presence of 2 mM HI-6. The results of this experiment shown in Figure 1, demonstrate that despite superior reactivation properties, F295L and F297I AChEs were not an improvement over wild-type AChE in detoxifying soman and sarin. On the other hand, E202Q AChE was 2-3 times more effective in detoxifying soman and sarin compared to wild-type AChE. These results suggest that the improved detoxification properties of E202Q AChE may be due to its reduced rate of aging compared to wild-type AChE. The combination of mutagenized enzymes with oxime-induced OP turnover suggests a promising future for the detoxification of OP compounds by enzyme bioscavengers.

The major requirements for an enzyme to be an effective bioscavenger for OP toxicity are: (a) relatively long half-life in circulation, (b) relatively high turnover number, (c) immunocompatibility, and (d) availability in sufficient quantities for use as a pretreatment drug. Therefore, in addition to the catalytic properties, the circulatory stability of these enzymes is imperative for their use as bioscavengers *in vivo*. Since glycan structures play a significant role in the circulatory stability of enzymes, we compared the glycan structures and pharmacokinetics of recombinant DNA-derived ChEs with plasma-derived ChEs.

HuS BChE contained the greatest amount of carbohydrate (31% by weight of protein) compared to other plasma-derived ChEs such as tFBS AChE and Eq BChE which contained 9% and 23% carbohydrate by weight, respectively. *Torpedo* AChE, mFBS AChE, rMo AChE, and rHu BChE contained 9%, 9%, 10%, and 13% carbohydrate by weight of protein, respectively (Table 2). The relatively high content of mannose suggested the presence of N-linked oligosaccharides, and the presence of N-acetylgalactosamine indicated the presence of O-linked oligosaccharides in all enzymes. The presence of galactose indicated that the majority of glycans in all ChEs except *Torpedo* AChE were of the complex or hybrid type rather than the high-mannose type. In addition,

substantial amounts of the oligosaccharides in all ChEs except Eq BChE were fucosylated. The total number of complex carbohydrate chains/subunit calculated from their mannose content for various ChEs are listed in Table 2.

Charge-based separation of the 2-aminobenzamide labeled oligosaccharides showed that they consist of neutral as well as acidic components (2). In all cases, no acidic oligosaccharides were detectable after neuraminidase treatment suggesting that the acidic substituent on the oligosaccharide chain was a covalently linked terminal outer-arm sialic acid residue.

Fractionation of the total pool of desialylated oligosaccharides by high resolution gel permeation chromatography, provided a basis for identifying the N-linked units present in various ChEs. One major (11.2 glucose units (gu)) and several minor distinct structural components for HuS BChE, Eq BChE, and tFBS AChE, were identified. The structures of these glycans were determined to be of the complex biantennary type (18,19). In contrast, size-based fractionation of the desialylated oligosaccharide pools yielded three-to-four major oligosaccharides for *Torpedo* AChE, mFBS AChE, rMo AChE, and rHu BChE. The glycans eluting at 11.2 gu and 12.2 gu are most likely of the complex biantennary type (19) and the other peaks probably correspond to high mannose, hybrid, tri- and tetra-antennary structures.

The molecular forms of various ChEs used in this study were determined by sucrose density gradient centrifugation analysis. HuS BChE, Eq BChE, and tFBS AChE, the three ChEs derived from plasma sources are tetrameric (t) in form (20,21), *Torpedo* AChE is dimeric in form (22) and mFBS AChE migrates as a monomer (m) (23). Of the two recombinant ChEs tested, rHu BChE is a mixture of monomers, dimers and tetramers, whereas rMo AChE exists in monomeric form only.

Following i.v. injection, the plasma activity of all ChEs declined in two phases, and curve fitting was carried out as described (12). In order to permit a meaningful correlation between the pharmacokinetic characteristics and the structural features of ChEs from various sources, we compared their circulatory properties by determining volume of distribution (V_{ss}), MRT, total body clearance (CL), and elimination rate constant (k_{el}), using a non-compartmental analysis (12). The results are summarized in Table 3.

V_{ss} was compared with the initial volume of distribution that is approximated by the plasma volume (V_p). A 1.1 to 1.4-fold increase in V_{ss} over V_p was observed for plasma-derived tetrameric forms of Eq BChE, HuS BChE, and FBS AChE. The less stable enzymes, mFBS AChE, rMo AChE and rHu BChE, clearly equilibrated with a slightly larger volume (V_{ss}/V_p _ 2).

Similarly the enzymes could be separated into two major groups according to their total body residence time. As indicated above for V_{ss}, one group contains the tetrameric forms of plasma-derived Eq BChE, HuS BChE, and FBS AChE, which are characterized by extended durations in the body (MRTs ~ 1902-3206 min). The second group contains mFBS AChE, rMo AChE, and rHu BChE, with MRT values of 205-304 min, which are 6- to 15-fold shorter than the tetrameric enzymes. MRT and V_{ss} were reasonably correlated with the molecular weight of the proteins that reflect the assembly of the ChE subunits, and with the fraction of sialylated oligosaccharides.

The molar ratio of sialic acid-to-galactose residues on HuS BChE, rMo AChE, and rHu BChE was ~1.0, suggesting that all the terminal galactose residues were capped with sialic acid (Table 2). However the MRT of HuS BChE was 9- and 14-fold greater than that of rMo AChE and rHu BChE, suggesting that the capping of galactose with sialic acid by itself is not sufficient to confer circulatory stability to ChEs. For tFBS AChE (MRT, 1902 min), Eq BChE (MRT, 3206 min), *Torpedo* AChE (MRT, 44 min) and mFBS AChE (MRT, 304 min), this ratio was ~0.5 (Table 2), suggesting that only half of the terminal galactose residues were capped with sialic acid, yet these enzymes greatly differed in their circulatory stability. These observations suggest that although the presence of sialic acid appears to be essential for maintaining ChEs in circulation, the location rather than the number of the non-sialylated galactose residues may be affecting circulatory stability.

CONCLUSIONS

The results presented here demonstrate that it is indeed possible to improve the detoxification properties of ChEs by site-directed mutagenesis. The combination of mutant enzymes and oxime reactivation, have very useful applications in many areas. An effective formulation can be developed for use in medical, surgical or skin decontamination of exposed subjects. Such formulations can also be used for decontamination of equipment, transportation vehicles and other environmental objects. This approach can be employed to develop effective methods for the safe disposal of stored nerve agents. The advantages of this approach will be relative ease of handling, cost-effectiveness, and relative safe disposal of detoxification products. In addition, the presence of residual AChE in the detoxifying media itself serves as an end point for completeness of the decontamination process.

To evaluate the possible use of rChEs as bioscavengers *in vivo*, we compared the mean residence time (MRT) in circulation of five tissue-derived and two rChEs i.v. injected in mice with their oligosaccharide profiles. The results

presented here reveal differences in the oligosaccharides of native and recombinant ChEs with regard to the total carbohydrate content and charge- and size-based oligosaccharide profiles. However, neither the carbohydrate composition nor the oligosaccharide profile could be completely correlated with the pharmacokinetic parameters of these enzymes. While the correlation between glycan characteristics and pharmacokinetic parameters is not fully understood, it is noteworthy that the glycans of recombinant ChEs and mFBS AChE displayed a remarkable heterogeneity in size and consist of hybrid and complex bi-, tri- and tetra-antennary structures. *Torpedo* AChE also contains high-mannose structures. The three plasma ChEs, on the other hand, contain mature glycans, which are predominantly of the complex biantennary type, confirming that these structures are responsible for the extended MRTs of the enzymes. The molar ratio of sialic acid-to-galactose residues on ChEs suggest that, location rather than the number of the non-sialylated galactose residues may be affecting circulatory stability. Site-specific analysis of glycan structures may elucidate the structures responsible for the rapid clearance of non-plasma ChEs and suggest suitable manipulations for improving the circulatory stability of rChEs.

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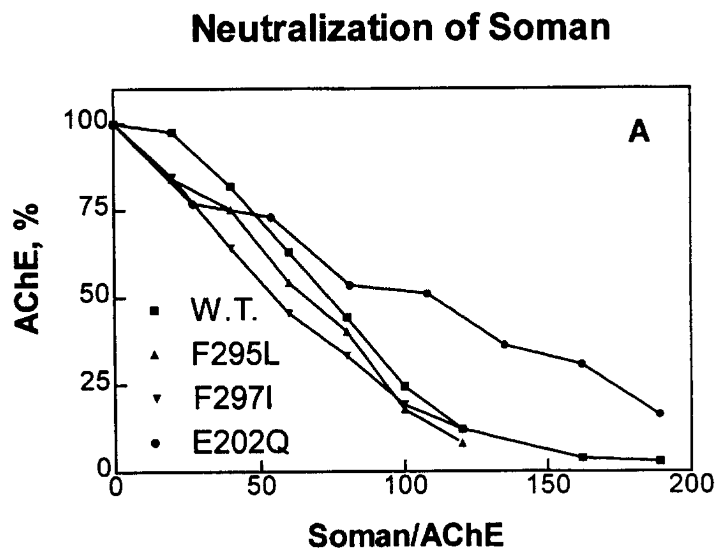
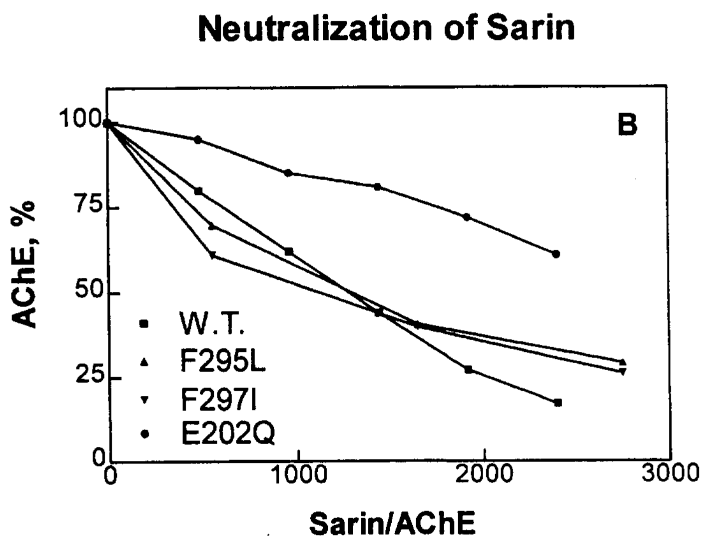
KEYWORDS

Bioscavenger, cholinesterases, site-directed mutagenesis, glycans, pharmacokinetics

FIGURES AND TABLES

Figure 1.





In vitro Detoxification of sarin or soman by Mouse Wild-Type and Mutant AChEs in the presence of HI-6. The reactivation of mouse wild-type (■), F295L (▴), F297I (▾) and E202Q (•) AChE (0.011 nmol) was carried out in the presence of 50 mM sodium phosphate, pH 8.0 containing 0.05 % BSA and 2 mM HI-6 after repeated additions of soman (1.8 nmol, Panel A) at 24-hr intervals, or san (0.275 nmol, Panel B) at 6-hr intervals. Residual enzyme activity was plotted against the cumulative amount of soman or sarin present in the reaction mixture. Data shown is representative of three experiments.

Table 1. Inhibition, reactivation, and aging Constants for recombinant mouse acetylcholinesterases

Inhibitor	Bimolecular rate constant $k \times 10^{-8}$ (M^{-1} min^{-1})			
	Wild-Type	E202Q	F295L	F297I
P(S)C(S)-Soman	1.20±0.27	0.200±0.017	0.99±0.01	0.10±0.007
P(S)C(R)-Soman	0.69±0.09	0.044±0.004	0.33±0.01	0.12±0.01
Sarin	0.08±0.03		0.11±0.02	0.07±0.01
	Second Order Reactivation rate constant for HI-6 k ($mM^{-1}min^{-1}$)			
	Wild-Type	E202Q	F295L	F297I
P(S)C(S)-Soman	0.50±0.07	0.05±0.01	2.10±0.46	0.60±0.15
P(S)C(R)-Soman	0.05±0.01	0.005±0.001	0.33±0.03	0.06±0.01
Sarin	0.51±0.06		0.66±0.04	2.60±0.32

Table 2. Number of glycans and the content of acidic oligosaccharides in cholinesterases

Enzyme	Mannose	N-acetyl glucosamine	Galactose	Sialic acid	Non-sialylated galactose[a]	Number of glycans[b]	Fraction of acidic oligosaccharides[c]
	nmol/nmol subunit						
Eq BChE	0	0	0	0	9	11	0
HuS BChE	0	51	0	0	2	12	0
tFBS AChE	9.5	0	0	3.5	0	3	0
Torpedo AChE	0	0	0	0	0	4	0
mFBS AChE	8.5	0	0	0	0	3	0
rMo AChE	0	0	0	0	0	3	0
rHu BChE	0	0	0	0	0	5	0

[a] Difference between galactose and sialic acid content
[b] Calculated from mannose content (three residues of this sugar per complex oligosaccharide)
[c] Calculated from area under the peaks (12)

91. WMD CASUALTY PLANNING

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INTRODUCTION

Since 1996, EAI Corporation has developed training, prepared plans, evaluated equipment and analyzed resources required for state and local response to terrorist incidents involving WMD. During the course of various efforts, it has become apparent that, although there are federal and private programs to support first responder training, there are few if any programs for emergency planners. This paper outlines some of the planning factors we use to develop WMD terrorism response plans and resource requirements.

DISCUSSION

The first step in planning for WMD terrorist incidents is for emergency planners to become more familiar with WMD hazards and limitations and the threat of WMD attacks. The terrorists do not need to be perfect, but they still need to be at least marginally capable or an attack is likely to create very few casualties.

Some hazards and limitations of WMD materials include the fact that:

- Biological materials, including various fatal diseases and toxins, can be produced fairly easily. However, variations between strains may result in weak or ineffective agents.
- Several common hazardous materials, such as Chlorine, are also considered chemical warfare agents. However, chemical warfare agents are significantly more toxic.
- Not all WMD materials are easy to make or obtain. Some manufacturing materials and equipment are subject to national or international regulation; purchase or theft can be tracked and reported. In addition, some agent manufacturing processes are very complex, and an incautious or incorrect step may kill or injure the terrorist or result in fairly impure agents.
- If the terrorists successfully purchase or steal WMD material they must still transport it, work with it to create the weapon they want, and store it until they are ready to use it. For example, although some biological agents are easy to manufacture, they are often very difficult to turn into useful weapons.
- Disseminating WMD materials can be difficult for several reasons. First, the dissemination device must not leak and must hold enough material to create the desired effect. Second, the device must be able to disperse the material as a dust, vapor, or aerosol in a useful particle size (typically 1 – 5 microns). Lastly, in an outside release, weather factors such as wind direction and speed, air stability, temperature, ultraviolet light, and precipitation will affect the persistency and spread of the material. If the release is indoors, weather factors are largely negated, but the terrorists must still account for air circulation and environmental controls.

Once planners understand the potential of WMD materials, they should identify and profile the hazards, prioritize the risk of an attack, create and apply scenarios to assess their vulnerability to attack, analyze the probable outcome of the attack, and determine the community's capability to respond to the attack. During this phase of the planning process, EAI uses threat information from various sources and develops planning factors concerning weapon types, probable WMD materials, attack methods, casualty estimation, and response equipment requirements.

For the Domestic Preparedness program, the FBI created a graph (see Figure 1) that broadly indicates the probability versus impact of some terrorist events. For example, it shows that while the probability of a terrorist detonating a nuclear device is low, if such an event were to occur it would have a devastating effect. It also shows that a chemical attack, while more likely, would have more limited affects and that a biological attack is both more probable and potentially as disastrous as a nuclear attack.

In their report, the Advisory Panel to Assess Domestic Response Capabilities for Terrorism Involving Weapons of Mass Destruction (Gilmore Panel) also addresses this probability versus consequence issue. In fact, they encourage readers to consider preparations for higher-probability/lower-consequence events.

Finally, in her recent book, "The Ultimate Terrorist," Jessica Stern discusses several of the same factors outlined by the FBI and the Gilmore Panel that make the higher consequence events less probable.

Based on these and other threat assessments, we developed the planning factors for chemical and biological agents shown in Tables 1 and 2. Table 1 identifies what we consider to be the most probable chemical and biological agents. Table 2 estimates casualty percentages.

Weapon Types: Worst-case scenarios involving the more likely types of WMD weapons are based on how rapidly the exposed victims present symptoms: fast-acting (minutes), slow-acting (hours), and long-delayed-acting (days or longer).

Fast-acting weapons will result in higher numbers of more seriously injured casualties on site. Slow-acting and long-delayed-acting weapons will initially present fewer symptomatic casualties but may result in an overall greater number of people exposed to the hazards. Traumatic wounds from the explosion or injuries such as broken bones suffered during the escape may also accompany all attacks involving WMD materials.

WMD Materials: Specific WMD agents were considered based on their notoriety, availability of the agent or precursors, ease of manufacture, ease of transport, ease of dissemination, toxicity or virulence, and availability of treatments. Figure 2 shows the relative toxicity of several chemicals, including many commonly used in industry.

Some highly toxic chemical agents are improbable for use in a terrorist incident. For example, Soman (GD), VX, and Lewisite (L) are not feasible due to the difficulty in acquiring precursors and synthesizing the agents. Phosgene Oxime (CX) is excluded due to its instability.

Some biological agents are not included because they have very low lethality (Q Fever, Venezuelan Equine Encephalitis) or lack effective therapies beyond supportive hospital treatment (viral hemorrhagic fevers, such as Ebola, and trichothecene mycotoxins). All a first responder may be able to do for victims exposed to these agents is provide limited medical care on site, decontaminate them, and transport them to a hospital rapidly for more definitive care.

Attack methods: In general, an attack will likely be a relatively unsophisticated event, using simple, low-technology dissemination methods with relatively poor results compared to military weapons.
The disseminator may be a homemade device that releases the agent by spraying or by breaking or exploding to splatter the target. The weapon material is likely to be a small quantity (less than 10 liters) of relatively impure agent (about 50%). The spread of the agent may be limited to a small radius around the device, exposing those closest to potentially lethal concentrations, while those at a distance would be exposed to significantly lower concentrations.

An indoor release is more likely to cause mass casualties. By clustering people together and removing weather considerations, an indoor attack may use much smaller quantities of agent to cause harm compared to an attack in an open space. An exception to this assumption may be an attack that results in the release of massive amounts of toxic industrial chemicals. For example, the accidental release of 45 tons of Methyl Isocyanate in Bhopal, India, in 1984 caused tens of thousands of casualties.

A covert dissemination of a delayed-acting biological agent would take days or weeks to manifest. Therefore, such attacks are unlikely to result in an emergency response to an incident involving casualties on site.

Casualty Estimation: Casualties expected from the various types of weapon systems are summarized in Table 2. For each category, the probable percentage of casualties resulting from the specified weapon may fall within the overall range for that category, leaning toward the upper (high), middle (mid), or lower (low) end of that expected range as indicated. These values are based on reviews of Department of Defense data and decades of experience in weapons testing.
Casualty status uses the Maryland Institute for Emergency Medical Services System categories listed below. Casualties in all categories may be present on site.

- Category 1 - immediate (red tag) - exposed with severe symptoms, in need of advanced lifesaving measures
- Category 2 - delayed (yellow tag) - exposed with mild symptoms
- Category 3 - minimal (green tag) - exposed/asymptomatic or not exposed
- Category 4 - expectant/deceased (black tag) - no further assistance needed

Casualty status may change rapidly depending on the WMD material involved. Up to one-third of the casualties initially triaged as category 2 or 3 may worsen on site to become category 1 or 2 respectively. This will increase the amount of equipment needed for support of these victims. Up to one-third of the category 1 casualties may die, although this will probably not reduce the amount of resources expected to be used in their care while living.

The numbers of fatalities (category 4 casualties) are relatively low for each agent class. As soon as the closest and most susceptible victims begin to show symptoms, the remainder of the population will attempt to exit the area. This may limit the numbers of individuals exposed to a lethal dose at the site of the attack.

Categories 1, 2, and 4 are higher for incidents involving trauma from an exploding device than for similar WMD attacks with non-explosive disseminators. Some people will be injured due to blast and fragmentation, others to trampling or falling during the panic to escape.

The victims of more conventional injuries may or may not be close enough to the release of the WMD materials to become casualties of the agent as well.

Response Equipment Requirements: Various federal offices have developed recommended lists of equipment to respond to a WMD incident. Typically, these list the type and kind of equipment, but not the number of items an agency should acquire.

We developed our recommended resource list to accommodate a planned response for 1000 total casualties. To determine the amount of equipment necessary to respond to an incident, we identify a specific agent and multiply the percentage of casualties in a given category by 1000.

SUMMARY

In general, planning for a WMD attack employs the basic All-Hazards Emergency Operations Planning methods. For planning purposes, a terrorist WMD event can be likened to a release of an extremely dangerous HAZMAT.

Casualties can be estimated by assessing the casualty producing properties of the most probable weapon types, WMD materials, and attack methods.

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KEYWORDS:
NBC, WMD, casualties, planning, terrorism

FIGURES AND TABLES

Figure 1.

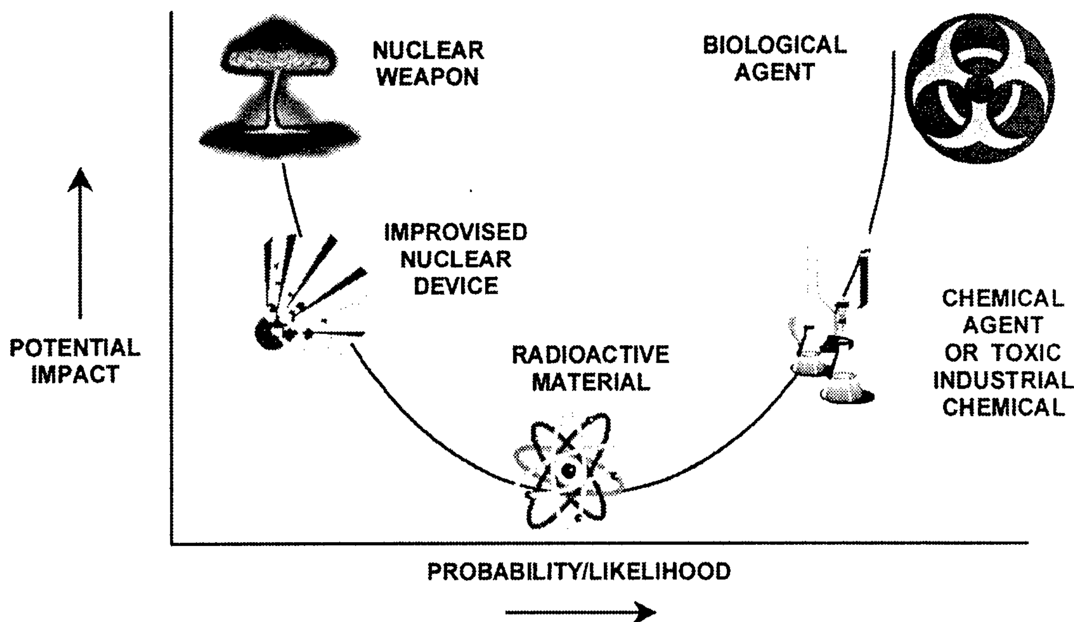

Table 1. Chemical and Biological Agents

MOST PROBABLE TOXIC CHEMICALS FOR TERRORISTS	**DOSE LETHAL TO 50% OF AN UNPROTECTED POPULATION IN MG MIN/M^3 & PPM**
ORGANOPHOSPHATE AGENT NAMES & (MILITARY DESIGNATIONS)	
TABUN (GA)	135 & 21
SARIN (GB)	100 & 17
PARATHION	6,000 & 504
CYANIDE AGENT NAMES & (DESIGNATIONS)	
HYDROGEN CYANIDE (AC)	4,000 & 3,620
CYANOGEN CHLORIDE (CK)	11000 & 4,275
PULMONARY AGENT NAMES & (DESIGNATIONS)	
PHOSGENE (CG)	3,200 & 790
CHLORINE (CL)	19,000 & 6,550
BLISTER AGENT NAMES & (DESIGNATIONS)	
SULFUR MUSTARD (H, HS, & HD)	1,500 & 230
NITROGEN MUSTARD (HN)	1,500 & 215
BIOLOGICAL TOXINS NAMES & (DESIGNATIONS)	
RICIN	24 & 0.009
BOTULINUM TOXIN (BOTTOX)	5 & 0.0008
STAPHYLOCOCCAL ENTEROTOXIN B (SEB)	5 & 0.0035
MOST PROBABLE BIOLOGICAL AGENTS FOR TERRORISTS	**INFECTIOUS DOSE (# OF ORGANISMS)**
BACILLUS ANTHRACIS (ANTHRAX)	8,000 - 10,000
YERSINIA PESTIS (PLAGUE)	100
FRANCISELLA TULARENSIS (TULAREMIA)	50

Table 2. Casualty Estimation

RATE OF ACTION	AGENT/MATERIAL	CASUALTY CATEGORY & (PROBABLE RANGE)			
		1 (1-15%)	2 (5-25%)	3 (50-90%)	4 (1-5%)
RAPID	ORGANOPHOSPHATE	HIGH	MID-HIGH	MID-LOW	HIGH
	CYANIDE	LOW	LOW	HIGH	LOW
	EITHER & TRAUMA	HIGH	HIGH	LOW	HIGH
SLOW	PULMONARY	MID	MID	MID	MID
	BLISTER	LOW	HIGH	MID	LOW
	BIOLOGICAL TOXIN	MID	MID-HIGH	MID	MID
	ANY & TRAUMA	HIGH	HIGH	MID-LOW	MID-HIGH
DELAYED	BIOLOGICAL PATHOGEN	LOW	LOW	HIGH	LOW
	EITHER & TRAUMA	MID-LOW	MID-LOW	HIGH	LOW

Ranges are + or - 5%; high, mid, or low are indicators of the number of casualties expected to occur within that percentage range. For example, if the range is listed as 5-25%, the word "high" means the trend will be towards 25% and the word "low" means

92. ELECTRONIC SYNDROMIC SURVEILLANCE FOR BIOTERRORISM AT THE WORLD TRADE ORGANIZATION MINISTERIAL CONFERENCE IN SEATTLE, WASHINGTON, 2000

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Timely, accurate, surveillance methods for the detection of disease due to agents of bioterrorism are critical for an effective public health response. We established an electronic system that could be quickly implemented in emergency departments (ED's) and would monitor patient visits for syndromes that may suggest bioterrorism during the World Trade Organization Conference (WTO).

Methods: The WTO Enhanced Surveillance Project (WTO-ESP) was comprised of three main components: a WTO - ESP data collection form to be completed for each patient seen in ED's of eight Seattle area hospitals, a laptop computer provided to each ED for remote data entry of information collected on the WTO - ESP form, and a central server which received the data from the WTO - ESP forms. Information from the WTO-ESP forms for all patients at each hospital were entered into the laptop computers by unit clerks. This information was transmitted to the central server and was monitored twenty four hours a day.

Results: A total of 10,577 reports were received from November 24, 1991 through December 10, 1999 from emergency departments in the participating Seattle area hospitals. Of these 10,577, 491 (4.6%) checked a box associated with a clinical syndrome and the remainder of the reports were either "none of the above" or, a box was not checked on the form.

Conclusions: Our surveillance system did not reveal any evidence of bioterrorism associated with this event in the Seattle area. The goals of the system were met - epidemiologists could immediately identify any conditions that warranted investigation and rapidly dispel rumors of illness related to natural or unnatural causes.

Keywords: bioterrorism, surveillance, epidemiology

93. ANTAGONISM OF ORGANOPHOSPHOROUS LETHALITY STERICALLY STABILIZED LIPOSOMES (SL) CONTAINING RECOMBINANT ORGANOPHOSPHOROUS ACID HYDROLASE (OPH)

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ABSTRACT
Sterically stabilized liposomes were employed as a carrier model to antagonize the lethal effects of the anticholinesterase organophosphorous agent, paraoxon. OPH rapidly catalyzes the hydrolysis of paraoxon to the less toxic 4-nitrophenol and diethylphosphate. This recombinant enzyme was encapsulated within SL and administered to mice either alone or in combination with pralidoximine (2-PAM) and/or atropine. These results indicate that this carrier model system provides a dramatic protective effects against the lethal effects of paraoxon. Moreover, when this carrier liposomes were administered with 2-PAM and/or atropine either alone or in various combinations, a striking synergistic protection against paraoxon was observed. The advantage of this new SL system is the high encapsulation efficiency and it can be stored at 2°C. SL containing recombinant OPH alone can protect better than 2-PAM and/or atropine combination against the lethal effect of paraoxon. Application of 2-PAM, atropine and SL containing OPH can protect over 1000 LD50 doses of paraoxon. Phosphotriesterase is being used to increase organophosphate (OP) degradation to phosphotriesterase to antagonize (OP) intoxication. For these studies, sterically stabilized liposomes encapsulating recombinant phosphotriesterase were employed. This enzyme was obtained from *Flavobacterium sp.*, and was expressed in *Escherichia coli*. It has a broad substrate specificity which includes parathion, paraoxon, and other organophosphorous compounds. Paraoxon is rapidly hydrolyzed by phosphotriesterase to the less toxic 4-nitrophenol and diethylphosphate. This enzyme was isolated and purified over 1600 fold and subsequently encapsulated within sterically stabilized liposomes (SL). The properties of this encapsulated phosphotriesterase were investigated. When these liposomes containing phosphotriesterase were incubated with paraoxon, it readily degraded the paraoxon. Hydrolysis of paraoxon did not occur when these sterically stabilized liposomes contained no phosphotriesterase. These sterically stabilized liposomes (SL) containing phosphotriesterases (SL)* were employed as a carrier model to antagonize the toxic effects of paraoxon by hydrolyzing it to the less toxic 4-nitrophenol and diethylphosphate. This enzyme-(SL) complex (SL)* was administered intravenously to mice either alone or in combination with pralidoxime (2-PAM) and/or atropine intraperitoneally. These results indicate that this carrier model system provides a striking enhanced prophylactic and therapeutic protection against the lethal effects of paraoxon. Moreover when these carrier liposomes were administered with 2-PAM and/or atropine, an enhanced protection occurred. (Supported by grants from NIH, NSF, USAMRDC, NOAA, and NATO)

INTRODUCTION
Parathion, an organophosphorous (OP) pesticide, is still as one of the most widely used insecticide worldwide and has caused toxicological problems. It is metabolized to its active metabolite, paraoxon, which is responsible for most of its activity and toxicity. Paraoxon exerts its toxic effect by inhibiting acetylcholinesterase (AchE). This would prevent the hydrolysis of the neurotransmitter, acetylcholine, and result in high accumulation of acetylcholine. These acetylcholine molecules bind to receptors causing overstimulation of the effector cells, resulting in cholinergic intoxication. Organophosphorous compounds are believed to exert their effect by phosphorylating the esteratic site of acetylcholinesterase, forming an almost irreversible complex. There are cholinesterase reactivators, i.e., pralidoxime (2-PAM) which can reactivate the OP-inhibited cholinesterase. This would increase the rate of dephosphorylation of the OP-inhibited enzymes, restoring the hydrolysis of acetylcholine. Atropine, a reversible OP pharmacologic antagonist, acts at the acetylcholine receptor, primarily at the muscarinic sites. One of the most common antidotal combinations now used to treat OP poisoning is atropine and pralidoxime. Atropine itself has severe autonomic side effects which can

affect stamina, vision, and body temperature control, etc. Pralidoxime (2-PAM), the biochemical antagonist, has limited absorption and poor physiological disposition factors. It would be desirable to have an organophosphorous antagonist which exerts its effect by actually destroying the toxicant. If there were a mechanism to degrade paraoxon rapidly as it enters the blood and before it reaches the target sites, paraoxon would possibly be much less toxic. Recombinant enzymes that rapidly hydrolyze OP compounds have been reported by (1) and (2), and have been described to protect animals against OP intoxications (3). However, the potential uses of such free enzyme preparations as antidotes are limited due to their unfavorable physiological dispositions and potential immunological reactions. These disadvantages can be partly overcome by encapsulating the enzyme within a bioprotective environment, i.e., sterically stabilized liposomes. The ability of the CRBC containing recombinant phosphotriesterase to antagonize the toxic and lethal effect of paraoxon was reported by (4). The mechanism of action of this carrier CRBC as an antagonist may be partly attributed to the rapid degradation of paraoxon by this enzyme. The phosphotriesterase is encapsulated within the stealth liposome and exerts no pharmacologic activity until the animal is exposed to the OP agent. Under those conditions, the OP agent is rapidly hydrolyze before it distributes to the target site.

Sterically stabilized, long circulating, stealth liposomes have found widespread use as model membrane systems, and have been extensively investigated for their potential use as drug carriers (5) and (6). First formulations of long circulating liposomes resulted from attempts to mimic some of the properties of the outer surface of red blood cells.

The present research describes the use of sterically stabilized liposomes as a carrier system for recombinant phosphotriesterase as a new approach to developing specific antidotes. The antidotal combination of 2-PAM and atropine are effective in antagonizing OP intoxication; however, the OP agent still remains in the body as neither of these antagonists actually destroys the organophosphates. Because of the potentially rapid action of OP compounds, it seemed appropriate that a major investigative effort be directed towards developing an agent to destroy the OP agent, rather than merely making the animal more resistant to the OP agent. This is being accomplished by encapsulating a phosphotriesterase enzyme within long circulating liposomes.

MATERIALS AND METHODS

Enzyme. A recombinant phosphotriesterase (EC 3.1.8) was purified from an *Escherichia coli* clone containing the plasmid expression vector pjK33 which was isolated from *Flavobacterium* sp (1)and (2). The enzyme was obtained in preparative amounts and purified over 1600 fold.

Encapsulation of Phosphotriesterase within Sterically Stabilized Liposome (SL).* Palmitoyloleoylphosphatidylcholine (POPC) and dipalmitoyl phosphatidylethanolamine-N-[poly(ethylene glycol)^{2000}] (PEG-PE) were purchased from Avanti Polar Lipid (Alabaster, AL). Purified cholesterol was obtained from Calbiochem (San Diego, CA). Lipids were stored in chloroform under argon at -70°C. Sepharose 4B was purchased from Pharmacia Fine Chemicals. Chloroform solutions of POPC (60 μmol), cholesterol (40 μmol) and PEG-PE (5.4 μmol) were mixed in a round-bottomed flask, and the solvent was removed slowly on a rotary evaporator at 37°C to obtain dry thin lipids film on the flask. Purified phosphotriesterase in HEPES [N-[2 - Hydroxyethyl] piperazine-N^{1}-[2-ethanesulfonic acid)]sodium salt] buffer (concentrated on Amicon Concentrator and clarified by centrifugation if necessary) was added to the dry lipids. The lipids film was hydrated slowly under argon by continuously rotating the flask on a rotary evaporator at 37°C for one hour. The milky liposome suspension was extruded sequentially through 0.2μ and 0.1μ polycarbonate membrane filter. Extrusion was repeated five times for each membrane to obtain a homogeneous size distribution of liposomes. Unencapsulated phosphotriesterase was separated from liposomes by gel filtration on sepharose 4B column. The encapsulation efficiency was calculated from the amount of encapsulated phosphotriesterase divided by the amount added times 100.

Phosphotriesterase Activity Determination in Sterically Stabilized Carrier Liposomes. Phosphotriesterase activity in liposomes was measured at room temperature, by determining the increase in p-nitrophenol concentration in the presence of excess paraoxon (4). One unit of phosphotriesterase is defined as that amount of enzyme which hydrolysed 1 μmol of paraoxon to p-nitrophenol per min.

Animals. Male Balb/C mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighting between 18-20 g were housed in room temperature and light controlled rooms, and were furnished with water and 4% Rodent Chow

(Teklad HSD, Inc., WI) *ad libitum*. All animal procedures were conducted in accordance with the guidelines by the NIH Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, DHEW Pub No. (NIH) 85-23, 1985).

In vivo experiments. Male mice received 5-10 units of phosphotriesterase intravenously (encapsulated within SL*) one hour prior to receiving paraoxon (in 6% cyclodextrin and propylene glycol solvent system) subcutaneously. The vehicle employed consists of 40% propylene glycol, 10 % ethanol and 50% water (v/v/v). Animals exposed to paraoxon with antagonists (atropine and/or 2-PAM and/or phosphotriesterase) were determined by 24 hr mortality. Surviving animals were observed for an additional one week for late developing toxicity. No gross toxic effects were apparent in mice receiving encapsulated phosphotriesterase, atropine, and 2-PAM, either alone or in various combinations. Atropine and 2-PAM were administered intraperitoneally to mice 30 and 15 minutes, respectively, prior to receiving paraoxon. The LD_{50} values with (SL)* were determined by the up-and-down method, and the estimated 95% confidence interval was determined by the method of Bruce, (13). For each experiment, 6-10 mice were used.

RESULTS

This is the first application of Sterically Stabilized Liposomes containing phosphotriesterase (SL)* to antagonize a toxicant. Genes for this phosphotriesterase enzyme had been identified from *Flavobacterium sp.* (12), and had been cloned and expressed in *E. coli.* Use of protamine sulfate treatment, ammonium sulfate fractionations, gel filtration and anion exchange chromatography resulted in the enzyme purification factor of over 1600. This purified phosphotriesterase was encapsulated into sterically stabilized liposomes.

This method for sterically stabilized liposome preparation resulted in approximately 80 % encapsulation efficiency. Phosphotriesterase entrapped in the carrier liposomes was capable of rapidly hydrolyzing paraoxon. Liposomes containing no enzyme, do not hydrolyze paraoxon. Enzymatic hydrolysis of paraoxon with various amount of (SL)* containing phosphotriesterase is shown. The reaction was linear at the time range and at the concentration range in which we conducted these experiments. Increases in p-nitrophenol formation was directly proportional to the amount of (SL)* containing phosphotriesterase. There was a direct linear relationship between the amount of (SL)* and the amount and rate of p-nitrophenol formed in the enzymatic reaction. No increase in p-nitrophenol concentration was detected when liposomes did not contain any enzyme.

Protective effects of phosphotriesterase encapsulated into sterically stabilized carrier liposomes (SL)* either alone, or in a combination of 2-PAM and/or atropine are summarized in Table 1, and are expressed as potency ratios (potency ratio= LD_{50} antagonized/LD_{50} unantagonized) to demonstrate the overall efficacy of these antidotal systems to antagonize the lethal effects of paraoxon. The encapsulated phosphotriesterase alone was more effective than the classic antidotal treatment of atropine and 2-PAM. Animals receiving both the encapsulated phosphotriesterase and 2-PAM or atropine showed dramatic increases in the protection, and it was even more highly enhanced when the encapsulated phosphotriesterase was used in combination with atropine and 2-PAM. The antidotal potency ratios, expressed as the protection of the antagonized paraoxon over unantagonized paraoxon, illustrate the dramatic enhanced effects of the (SL)* system in antagonizing paraoxon intoxication not only alone, but particularly in combination with atropine and/or 2-PAM. The LD_{50} value of paraoxon of the control animal was used as a potency ratio of 1.0. (SL)* alone caused an increase in the potency ratio to 139.0. When 2-PAM (potency ratio=5.0) was used in a combination with (SL)*, the potency ratio was increased to 611.3. Atropine (potency ratio=2.3) used in a combination with (SL)* exhibited an increase in the potency ratio to 600.3. When both atropine and 2-PAM (potency ratio=61.1) were used in a combination with (SL)*, the potency ratio was elevated to 1022.

DISCUSSION

This research is an attempt to use fast catalytic enzymes encapsulated within biodegradable carriers to antagonize the lethal effects of toxicants. These results represent the first attempt to use sterically stabilized liposomes containing recombinant phosphotriesterase (SL)* to hydrolyze paraoxon. Hopefully this approach may serve to protect against the toxic effects of other chemicals with other enzymes. This is being accomplished by encapsulating recombinant enzyme with high catalytic activity within a sterically stabilized liposome. This phosphotriesterase has high affinity to paraoxon (K_m=0.05 mM); therefore, it is a good substrate to study OP antagonism on this enzyme. There is no significant

difference in Km values between the free and the encapsulated phosphotriesterase; therefore paraoxon readily penetrates through the membrane of the carrier cells, -what is a critical requirement for the application of our approach.

Phosphotriesterase is being encapsulated within (SL)* to accomplish several factors. First, this places the phosphotriesterasein a bioprotectiveenvironment so that the enzyme will not be rapidly degraded in the body. Secondly, this prolongs the activity of the enzyme which is important as the OP agent is lipid soluble and can remain in the fatty depot for a long time. In order to remove the OP agent, an antagonist with long duration of action is desirable. Thirdly, the mechanism of action of this antagonist is quite different from the classic antagonist of 2-PAM and atropine. The combination of the latter two antagonists merely prevent the pharmacologic actions of the OP agent by making the animals more resistant to the OP agent, but the toxic agent still remains in the body. By the use of phosphotriesterase within (SL)*, the OP agent could be degraded in the body, thereby terminating its action. OP hydrolyzing enzymes have been found in various human and animal tissues (9) and (10) and bacteria (11). When phosphotriesterase preparation is administered intravenously, it can protect animals against OP poisoning (12) and (3) but the enzyme is rapidly; therefore, the therapeutic value of the free enzymes alone as an antagonist is very limited.

This new conceptual approach which uses carrier cells containing highly purified recombinant enzymes to rapidly destroy the organophosphorous (OP) compound before it reaches the target site provides striking results. Although the free purified enzyme preparations can protect animals against certain OP intoxications, their clinical uses are very limited in scope due to their rapid degradation and elimination by body defense systems. The half life of free peptide preparations is only about 20 minutes; therefore, it is impractical to use them to prevent or to treat exposures. The encapsulation of the enzyme of interest into carrier cells, enhances the stability of the enzyme and prolongs the life-span of the carrier cells. Once the phosphotriesterase encapsulated into a carrier cell is administered to the body, the enzyme within the carrier cells could continue to hydrolyze toxic OP agents. The cell carrier system may be useful not only in prevention and/or treatment of acute OP toxicity, but also it may prevent the delayed toxicity; in this case, preventing toxicities from chronic exposure to OPs, which would not be achievable by other approaches. This could be particularly important to agricultural workers and pesticide manufacturing personnel. It may also play a significant role for military personnel as the OP nerve gases are one of the most potent chemical weapons. It permits a practical prophylaxis in our combat troops as the protection can last for days to months, depending on the carrier system, without affecting the mobility and physical performance of our troops. Furthermore, this cell carrier system may be useful in combination with the traditional antidotes, atropine and 2-PAM, since the encapsulated phosphotriesterase protects by an entirely different antidotal mechanism.

This approach has developed a prophylactic agent which is superior to the antidotal combination of 2-PAM and atropine. This study indicates that the magnitude and duration of protection could be greatly increased when highly purified phósphotriesterase was entrapped into sterically stabilized liposomes. The (SL)* allows permeation of the paraoxon molecules to freely enter and leave the carrier vehicle cells reaching a rapid equilibration and resulting in a rapid detoxication when the enzyme still retains its activity in the protected intracellular environment.

The role of 2-PAM and/or atropine in this marked synergism is still not clear. For example: if the acetylcholinesterase inside the nervous system plays a major role, the 2-PAM has to distribute to the nervous tissues. 2-PAM has an electrical charge of 1, it is unlikely that it can penetrate the blood brain barrier. It should form a neutrally charged structure to do this. One possible explanation for the OP antagonism is, that the peripheral tissues outside the vascular system probably plays the major role of the OP intoxication. It should be noted, that the phosphotriesterase in combination with atropine make up the most striking potentiation. Whereas 2-PAM is enhanced over 100 fold with phosphotriesterase, atropine is enhanced over 200 fold. Since atropine occupies predominantly the muscarinic receptors for acetylcholine, the mechanism involved the reactivation of inhibited cholinesterase must still play a major role. To explain this dramatic protection probably does involve a series of factors. For example, to assume the construction of the OP inhibited acetylcholinesterase is partially reactivated, and the lowering of the acetylcholine concentration will permit the atropine to act at a more enhanced level. Since the atropine and acetylcholine compete for the receptor occupation, with the decrease in acetylcholine, the role of atropine becomes more prominent. An enzyme with a high turnover number may be necessary for these dramatic protections observed. With lower turnover number the dramatic enhancement may not be apparent, as the cascade of events would not be accelerated.

In summary, a new concept has been presented for the antagonism of OP intoxication. This general approach has great conceptional significance, as it suggests the potential for encapsulating other enzymes as a drug delivery system

to degrade other chemical toxicants.

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KEYWORDS

Phosphotriesterase, OPA anhydrase, paraoxonase, paraoxon antagonist, paraoxon hydrolysis, sterically stabilized liposomes, stealth liposomes, long circulating liposomes

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FIGURES AND TABLES

Table 1. Effects Of 2-Pam, Atropine And Phosphotesterase Encapsulated Within SL On The LD_{50} Of Paraoxon In Mice

Experiment # Treatment before paraoxon[a]	LD_{50} (mg/kg)[b]	Potency Ratio[c]
1. Control	0.9 (0.57-1.23)	1.0
2. Atropine	2.08 (1.51-2.84)	2.31
3. 2-PAM	4.50 (3.28-6.16)	5.0
4. SL	125.2 (91.39-170.5).	.0
5. Atropine + 2-PAM	55.0 (40.15-68.5)	61.1
6. Atropine + SL	540.3 (394.5-740.21)	600.3
7. 2-PAM + SL	550.2 (401.6-753.77)	611.3
8. 2-PAM + Atropine + SL	920.0 (671.6-1260.4	1022.2

a Paraoxon (0.6 to 1,200 mg/gk) was delivered subcutaneously to mice in 6% cyclodextrin and/or propylene glycol solvent solutions. The Propylene glycol solvent consisted of 40% propylene glycol, 10% ethanol, and 50% water. Atropine sulfate (10 mg/kg) and 2-PAM-Cl (90 mg/kg) was given intraperitoneally 30 and 15 minutes respectively to receiving paraoxon. SL (0.2-0.4 mL/mice) was intraveneously through tail vein 1 hr prior to receiving paraoxon.

b LD_{50} values were determined by the up and down method (simulated up-and-down study, Dixon, 1965), and the estimated 95% confidence interval was determined by the method of Bruce, (1985). For each experiment, 6-10 mice were used. The LD_{50} values were calculated from the equation: $\log(LD_{50}) = \log(Dose_{final}) + k \log(d)$, where dose final is the final dose administered, k is the tabular value from table (Dixon, 1965), and d is the interval between doses.

c

$$\text{potency ratio} = \frac{LD_{50} \text{ of paraoxon - antagonized}}{LD_{50} \text{ of paraoxon - unantagonized}}$$

94. EFFICACY OF OXIMES AND ADAMANTANES AGAINST SOMAN POISONING IN MICE

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INTRODUCTION

The "classical" nerve agents, such as tabun, sarin, soman, and VX are extremely toxic cholinesterase inhibiting organophosphates. The majority of currently available oximes are quite effective in reactivating cholinesterase inhibited by sarin, VX, and depending on the oxime, tabun. Of many oximes, HI-6 is most effective in restoring soman-inhibited acetylcholinesterase activity, However, despite this, the treatment of soman has not been completely solved (1,2). In contrast, besides the fact that HI-6 is clearly the oxime that is the most effective in restoring soman-inhibited acetylcholinesterase activity, the treatment of soman intoxication still represents a problem that has not been completely solved (1,2). Soman-inhibited acetylcholinesterase is very difficult to reactivate following poisonings with multiple lethal doses of soman. In addition, the soman-acetylcholinesterase complex "ages" very rapidly making it resistant to reactivation and does not undergo spontaneous reactivation. Also, convulsive activity in soman intoxication creates a problem that has been linked to irreversible brain damage. Some experiments have shown that memantine (Mem), 1-amino-3, 5-dimethyl adamantane, prophylaxis potentiates the therapeutic activity of standard antidotes used in organophosphate intoxications (3-7). Therefore, the possible antidotal interactions of anticonvulsive drug, memantine as well as its active and less toxic metabolite, 1-amino-3-hydroxymethyl-5-methyl adamantane (Mrz 2/373) and oximes were tested in mice poisoned with soman.

MATERIALS AND METHODS

Chemicals. Soman and oximes - pralidoxime (PAM-2), trimedoxime (TMB-4), obidoxime (LüH-6) and HI-6 - were obtained from the Military Medical Academy, Belgrade, Yugoslavia. Soman was 98.5% pure and oximes, analyzed using HPLC technique were greater than 99% pure. Memantine and Mrz 2/373 were kindly supplied as a gift by Dr. Guenter Quack, from Merz+Co, GmbH & Co., Frankfurt/M, FR Germany. All the other chemicals of analytical grade were obtained from Merck AG (Darmstadt, FR Germany). Soman stock solution was prepared in isopropanol. Oximes were dissolved in distilled water and diluted to the required concentration immediately prior to use. (Was the soman purity determined from HPLC?)

Animal experiments. Male albino mice (18-24 g) were obtained from the Military Medical Academy, Belgrade, Yugoslavia. The mice were acclimatized at least one week prior to use and received food and tap water *ad libitum*. All tested substances were administered intravenously via the tail vein at a volume of 0.1 mL/20 g body mass. Experimental animals were pretreated *iv* with oximes, memantine, Mrz 2/373 as well as with combinations of oxime and memantine or Mrz 2/373, at different time intervals (1-60 min) before 1.3 LD-50 (63.70 μg/kg; 0.35μmol/kg) soman *iv*. The dose of memantine and Mrz 2/373 was fixed at 10 mg/kg. Median effective doses were calculated according to the method of Litchfield and Wilcoxon (8), with 95 % confidence limits. These data were used to calculate ED-50 at null time (ED-50_0) and efficacy half time ($t_{1/2}$ eff.), i.e., half time of the ED-50 decrease.

In a separate experiment, the brain, diaphragmal and erythrocyte acetylcholinesterase (AChE) and plasma carboxylesterase (CarbE) activities were determined. The dose of HI-6 equivalent to the dose of ED-50_5, i.e. the dose of HI-6, which protects 50% of the poisoned animals when administered 5 min before 1.3 LD-50 of soman (3.1 mg/kg; 7.96 _mol/kg) and/or adamantanes (10 mg/kg) were administered 5 min before the animals received soman 0.75 LD-50 (36.75 _g/kg; 0.20 _mol/kg). Mice were decapitated and exsanguinated 5 min following soman poisoning. Diaphragm and brain were removed and homogenised in isotonic saline. The enzyme activity in brain and diaphragm was measured by the spectrophotometric method (9) and erythrocyte acetylcholinesterase was determined titrimetricaly (10) using acetylthiocholine iodide as substrate. Plasma was assayed for CarbE by the method of Boskovic et al. (11) using tributyrin as substrate.

Data analysis. Statistical significance was determined by means of Student's t-test and Mann-Whitney U-test, and differences were considered significant when $p < 0.05$.

RESULTS

Calculated ED-50_0 values at null time of memantine, pralidoxime, trimedoxime, obidoxime and HI-6 were 39.98, 329.57, 4.65, 25.78 and 7.98 _mol/kg, respectively (Table 1). These doses are expected to keep 50 % of the animals alive, if oxime and soman are administered simultaneously. Mrz 2/373 did not counteract the toxic effects of soman at any time of pretreatment. Along the tested time intervals, HI-6 afforded the best protection for experimental animals. Efficacy half times increased according to the following order: trimedoxime < pralidoxime < obidoxime < HI-6 < memantine. Co-administration of memantine significantly decreased the ED-50_0 of pralidoxime (7.67 times), obidoxime (9.34 times) and HI-6 (4.45 times) (Table 2). When trimedoxime was applied with memantine, no changes of its antidotal effect could be seen. However, in mice pretreated with trimedoxime, its efficacy half time was increased by 4.82 fold.

Following administration of soman alone, mice still had 44.53 % of their diaphragmal, 28.07 % of their brain and 4.67 % of erythrocyte acetylcholinesterase functional. When relatively low dose of HI-6 (3.1 mg/kg) was applied, acetylcholinesterase activity increased in all tissues examined (Figure 1). The results obtained in diaphragms showed that administration of either HI-6 or memantine provided significant recovery of diaphragmal acetylcholinesterase activity. In mice pretreated with the combination of HI-6 and memantine, diaphragmal acetylcholinesterase activity was even higher than in mice, which received HI-6 only. Among tested tissues, the highest recovery of inhibited acetylcholinesterase was obtained in the diaphragm. Co-administration of adamantanes (memantine, Mrz 2/373) produced a statistically significant increase in erythrocyte acetylcholinesterase activity. In all the prophylactic regiments used, plasma carboxylesterase activity was higher than after soman alone, but yet not significantly different.

DISCUSSION

The results presented in numerous previous studies demonstrate that HI-6 is the least toxic and most efficacious oxime examined as an antidote of soman poisoning in mice. The lack of or low antidotal action of the other oximes is due to their relatively low reactivating potency combined with relatively high toxicity. Until now, it was undoubtedly shown that HI-6 provided best protection and reactivating effect in soman poisoning (12, 13). A comparison of the various congeners of HI-6 illustrated that an oxime group and an amide group were essential for therapeutic activity. The oxime group in position two of the first pyridinium ring was required for reactivation of soman-inhibited acetylcholinesterase and the amide group in position four of the second pyridinium ring was required to reduce the inherent toxicity of the bispyridinium oxime structure (14, 15).

In our experiment, administration of HI-6, five minutes before soman, provided a significant increase of brain and especially diaphragmal acetylcholinesterase activity. For many years, it has been assumed that if an oxime could reactivate the organophosphate-inhibited acetylcholinesterase in the CNS, a beneficial effect should result. However, various studies have reported results, which do not confirm this hypothesis. Clement (16) found that mice, which died following soman poisoning, had higher brain cholinesterase activity than those which survived soman poisoning by the prophylactic administration of HI-6. This and other similar results question the significance of reactivation of soman-inhibited acetylcholinesterase in the CNS and suggest that inhibition of acetylcholinesterase in the CNS is not the primary lesion (?) in the lethality produced by soman. Having in mind the fact that the greater extent of acetylcholinesterase reactivation was seen in diaphragm than in brain, it could be assumed that reactivation of diaphragmal acetylcholinesterase was more important in the beneficial therapeutic action than reactivation of central acethylcholinesterase in mice poisoned with soman.

Although co-administration of memantine produced a decrease of ED-50 doses for all the oximes used, with the exception of trimedoxime, special attention was paid to its combination with HI-6, as the most relevant one in the case of soman poisoning. It was already shown that memantine in combination with atropine can prevent or antagonize the acute as well as subacute anticholinesterase poisoning due to carbamate or organophosphate (3-7). Also, it was suggested that memantine could counteract the effects of anticholinesterases by protection of acetylcholinesterase from inhibition, rapid reactivation of inhibited acetylcholinesterase, protection of carboxylesterase, and by speeding-up the bioelimination of the poison. Stojiljkovic (17) found that memantine and its metabolite (Mrz 2/373) along with atropine, HI-6 and diazepam provided better prophylaxis than physostigmine or pyridostigmine in soman-intoxicated rats. Co-administration of HI-6 and Mrz 2/373 produced some recovery of the enzyme activity in all the groups and tissues tested, but since a significant difference from soman group was achieved only in erythrocyte acetylcholinesterase activity, it was concluded that obtained results were not fully in accordance with those previously cited. Some of these discrepancies could be explained by differences in experimental design. The other explanation for variance of Mrz 2/373 efficiency observed in mice and rats could be ascribed to interspecies differences in importance of carboxylesterases for detoxification of soman (18). The major prophylactic influence of memantine and its combination with HI-6 was observed in acetylcholinesterase activity in

peripheral tissues. In accordance with this finding, it was recently published that in the treatment of acute carbofuran poisoning, memantine provided complete protection and reversal of the induced biochemical changes in diaphragm muscle by preventing depletion of high-energy phosphates and maintaining normal cell membrane characteristics, including permeability and integrity (19).

Soman exposure can lead to irreversible lesions of the central nervous system. Numerous studies have demonstrated that the excitatory amino acid glutamate plays a prominent role in the maintenance of soman induced seizures and in the subsequent neuropathology via an overstimulation of glutamatergic N-methyl-D-aspartate (NMDA) receptors. Memantine is known as a substance with NMDA antagonistic properties (20-22). Therefore, memantine NMDA receptor blocking anticonvulsant properties should be also included in its prophylactic potency against soman.

Application of memantine, along with the oximes used in this study, significantly lowered the oxime doses necessary to protect experimental animals from soman toxicity. Among the mechanisms explained, our results suggest that its efficacy could be ascribed to the combination of the protection of acetylcholinesterase activity in some vital tissues, such as diaphragm, and direct antagonistic effect of memantine on NMDA receptors in the brain of mice poisoned with soman.

SUMMARY

The aim of this study was to investigate the efficacy of the four pyridinium oximes - pralidoxime (PAM-2), trimedoxime (TMB-4), obidoxime (LüH-6) and HI-6 - alone or in the combination with memantine and its principal metabolite 1-amino-3-hydroxymethyl-5-methyl adamantane (Mrz 2/373) against soman in mice.

Male Albino mice were pretreated *iv* with oximes and adamantanes at various times before 1.3 LD-50 of soman *iv* in order to obtain their $ED\text{-}50_i$. In a separate experiment, the brain, diaphragmal and erythrocyte acetylcholinesterase and plasma carboxylesterase activities were determined after sacrificing mice 5 min after soman 0.75 LD-50. Oxime HI-6 (ED-50 value at 5 min before soman) and adamantanes were administered 5 min before soman. In the combination regimens and in the biochemical experiments the dose of memantine and Mrz 2/373 was fixed at 10 mg/kg *iv*.

Along tested time intervals, HI-6 afforded the best protection of experimental animals, and calculated $ED\text{-}50_0$ value of HI-6 was 7.96 _mol/kg. Memantine significantly (up to 9 times) decreased $ED\text{-}50_0$ values of all the oximes used, with the exception of TMB-4. Among tested tissues, the highest recovery of inhibited acetylcholinesterase was obtained in the diaphragm. Co-administration of adamantanes (memantine, Mrz 2/373) produced a statistically significant increase in erythrocyte acetylcholinesterase activity. It could be concluded that memantine antidotal efficacy could be ascribed to the protection of acetylcholinesterase activity.

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KEYWORDS
Soman, memantine, adamantanes, oximes, HI-6, mouse, acetylcholinesterase, carboxylesterase.

FIGURES AND TABLES

Figure 1. Acetylcholinesterase and carboxylesterase activities in mice following soman poisoning: effect of HI-6 and/or adamantanes

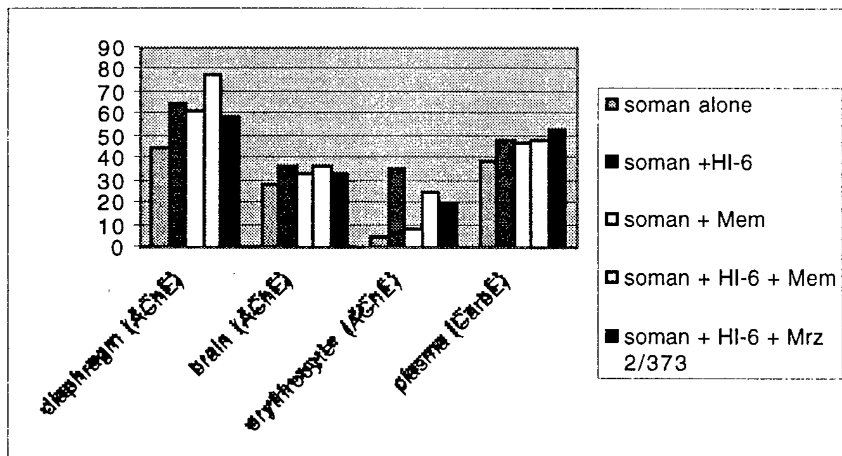

Table 1. Efficacy data: oximes and memantine in mice poisoned with soman

	Mem	PAM-2	TMB-4	LuH-6	HI-6
time (min)	ED-50_t (mmol/kg)				
1		370.76			
2		417.10	13.35		
5	45.47		64.82	35.63	8.63
12.5	52.05			64.70	
20	64.84			100.28	21.44
40					48.30
60					93.45
	ED-50_0 (mmol/kg)				
	39.98	329.57	4.65	25.78	7.96
	$t_{1/2}$ eff.(min)				
	28.88	5.88	1.31	10.09	16.24

Table 2. Efficacy data: combinations of oximes and adamantanes in mice poisoned with soman

	PAM-2 + Mem	TMB-4 + Mem	LuH-6 + Mem	HI-6 + Mem	HI-6 + Mrz 2/373
time (min)	ED-50_t (mmol/kg)				
1	74.27				
2	129.07				
5		6.16	4.70	2.56	23.47
12.5		28.55			
20		59.62	21.07	26.22	91.36
27.5		74.82			
40			174.88	150.99	138.86
	ED-50_0 (mmol/kg)				
	42.95	5.00	2.76	1.79	23.08
	$t_{1/2}$ eff.(min)				
	1.26	6.31	6.71	6.02	14.15

95. THE PROTECTIVE EFFICACY OF CALCIUM CHANNEL BLOCKERS IN SOMAN POISONED RATS

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INTRODUCTION

Organophosphorus anticholinesterase inhibitors are important insecticides, which account for a number of deaths every year and furthermore, stock piling these agents as potential weapons represents an ongoing societal concern. The mechanisms of their toxic action appear to involve at least two processes: a) the irreversible phosphorylation of cholinesterase leading to acetylcholine accumulation at central and peripheral cholinergic neuroeffector junctions (1) and b) the induction of presynaptic repetitive discharges leading to enhanced acetylcholine release and consequently augmented postjunctional responses (2). Calcium channel blockers (CCBs) have been shown to suppress such discharges and the accompanying fasciculation, and accordingly, may influence the organophosphate toxicity (3).

CCBs represent not only chemically, but functionally heterogenous group with various binding sites on calcium channels both in peripheral tissues and in neuronal structures (4). The present experiments were designed to determine whether structural differences among CCBs as well as the time of their application in relation to that of soman, a highly toxic organophosphorus chemical warfare nerve agent, may influence the acute soman toxicity and efficacy of its standard antidotes in rats.

MATERIAL AND METHODS

Adult male Wistar rats weighing 180 to 220 g were used in all experiments. Tested drugs were freshly prepared as follows: atropine sulphate ("Sigma"), oxime HI-6 ("Bosnalijek", Sarajevo), verapamil ("Lek", Ljubljana) and diltiazem ("Alkaloid", Skopje, injection vial of 25 mg liophilizate) were dissolved in saline. Nicardipine ("Lek", Ljubljana) was dissolved in 10% (v/v) ethanol. Diazepam was used from commercially available parenteral solution ("Apaurin" ampoules, "Lek", Ljubljana). All solutions were prepared in concentrations so that 0.1 ml contained the amount of substance to be given per 100 g body weight. The doses of drugs used were: atropine 10 mg/kg, oxime HI-6 10 mg/kg, diazepam 2.5 mg/kg, verapamil 10 mg/kg, nicardipine 10 mg/kg and diltiazem 25 mg/kg. CCBs (verapamil, diltiazem and nicardipine) and soman were administered by *sc* injection, and standard antidotes (atropine, oxime HI-6, diazepam) via *im* route. CCBs were given either 30 minutes before or immediately after application of soman. Standard antidotes were always injected immediately after soman administration. The median lethal doses (LD-50) of soman in absence or presence of CCBs and standard antidotes were calculated on the basis of 24-hour survival of poisoned animals and served for evaluation of protective efficacy of drugs given (5).

RESULTS AND DISCUSSION

Efficacy of CCBs tested, given alone or in combination with standard antidotes, in protection of rats acutely poisoned with soman, was presented in Table 1 and 2, respectively.

Among CCBs tested only verapamil (phenylalkylamine derivative), given either before or immediately after soman, produced significant protective effect (Table 1), but without enhancing the protective effect of standard antidotes (Table 2). Nicardipine (1,4-dihydropyridine derivative) failed to offer the protection in poisoned animals (Table 1) and, moreover, lowered the protective efficacy of standard antidotes (Table 2). Diltiazem (benzothiazepine derivative) alone produced significant protective effect only when given as pretreatment (Table 1), but failed to increase the protective efficacy of standard antidotes, even reduced it when given immediately after soman (Table 2).

Similarly to our work Dretchen et al. (6) were used various CCBs (verapamil, diltiazem and three different dihydropyridine derivatives, nifedipine, nitrendipine and nimodipine) in protection against DFP toxicity in the mouse. Given 30 minutes before the organophosphate only verapamil and dihydropyridine derivatives provided significant protection against the lethal effects of DFP. However, in combination with standard antidotes atropine and oxime 2-PAM, only verapamil and nifedipine produced a still higher level of protection (6). Karlsson and Sellstr^m (7) also reported that nimodipine was effective when used as a pretreatment, alone or in combination with atropine and pyridostigmine, in mice acutely poisoned with soman. These results, except those related to verapamil, are in contrast to our ones. In the present study nicardipine, a member of dihydropyridine CCBs, failed to offer any protection regardless the time of administration in relation to that of soman, and, even, reduced the protective effect of standard antidotes when given in combination with them. This finding is similar to that of Milovanovic et al. (8) which used nicardipine and nimodipine, alone or in combination with standard antidotes (atropine, oxime HI-6, diazepam), in protection of mice intoxicated with soman. Both CCBs, administered immediately after the poison, enhanced soman toxicity and reduced protective efficacy of the antidotes given (8).

It is very difficult to interpret these results at the moment. In above mentioned studies various CCBs have been used in protection against various organophosphates, in different species, and in different both dose and treatment regimens. Besides, different CCBs have different affinity to the binding sites on calcium channels in different tissues. The site of action of verapamil and diltiazem is the intracellular domain of the calcium channel, whereas dihydropyridine CCBs produce their action via the extracellular domain of the channel (4). Furthermore, verapamil and diltiazem have stronger affinity to the cardiac tissue, and dihydropyridine CCBs to the vascular smooth muscle. Corbier and Robineau (9) reported that inhibition of Na^{+}/K^{+}ATPase by an organophosphate compound, methylphosphonothiolate, could lead to ventricular arrhythmia that is proposed to be caused by intracellular calcium overload. It has been demonstrated that soman may contribute partially to the overload by reducing intracellular calcium uptake into microsomes and mitochondria (10). Having in mind these facts it could be concluded that the protection against soman obtained in our study with verapamil and, partially, with diltiazem might be a consequence of cardioprotective action of these CCBs. Our earlier results demonstrated the excellent cardioprotective effect of verapamil in soman-intoxicated rats (11).

CONCLUSION

Our results suggest that both the protective efficacy of CCBs in soman-poisoned rats and their influence on protection afforded by standard antidotes might be, at least in part, dependent on their chemical structure as well as the time of their administration in relation to that of soman.

SUMMARY

This study was designed to determine whether structural differences among calcium channel blockers (CCBs) as well as the time of their application in relation to that of soman may influence the acute soman toxicity and efficacy of its standard antidotes in rats.

The experiments were performed on adult, male Wistar rats. CCBs, phenylalkylamine derivative verapamil (10 mg/kg), 1,4-dihydropyridine derivative nicardipine (10 mg/kg) and benzothiazepine derivative diltiazem (10 mg/kg), were injected *sc* 30 minutes before or immediately after soman (*sc*), alone or aloÓg with atropine (10 mg/kg *im*), oxime HI-6 (10 mg/kg *im*) and diazepam (2.5 mg/kg *im*), always administered immediately after the poison. The median lethal doses of soman with or without CCBs and standard antidotes were calculated on the basis of 24-hour survival of poisoned animals and served for evaluation of protective efficacy of drugs given.

Among CCBs tested, only verapamil, given either before or immediately after soman, produced significant protective effect, but without enhancing the protective effect of standard antidotes. Nicardipine failed to offer the protection in poisoned animals and, moreover, lowered the protective efficacy of standard antidotes. Diltiazem alone produced significant protective effect only when given as pretreatment, but failed to increase the protective efficacy of standard antidotes, even reduced it when given immediately after soman.

These results suggest that protective efficacy of CCBs in soman-poisoned rats and their influence on protection afforded by standard antidotes might be dependent on their chemical structure as well as the time of their administration in relation to that of soman.

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KEYWORDS

nerve warfare agents, soman, calcium channel blockers, verapamil, nicardipine, diltiazem

FIGURES AND TABLES

Table 1. Protective efficacy of various calcium channel blockers against soman toxicity in rats

Pretreatment time (min)	LD-50 of soman	(95% Conf. Limits)	Protective index (PI)
-	0	(90.56-108.19)	1
30	0	(104.92-215.78)	1.52*
0	0	(104.92-215.78)	1.52*
30	0	(184.13-326.63)	1.67*
0	0	(45.53-213.80)	0
30	0	(45.53-213.80)	0
0	0	(45.53-213.80)	0

$$PI = \frac{\text{LD-50 of soman in treated animals}}{\text{LD-50 of soman in the control}}$$; *p<0.05 *vs* the control

Table 2. Protective efficacy of various channel blockers given in combination with standard antidotes (atropine + oxime HI-6 + diazepam) against soman toxicity in rats

Treatment	Pretreatment time (min)	LD-50 of soman	(95% Conf. Limits)	Protective index (PI)
Control	-	0	(90.56-108.19)	1
Standard antidotes	0	0	(117.79-292.98)	1.88*
Verapamil (10 mg/kg sc) +Standard antidotes	30	0	(113.04-255.37)	1.56*
	0	0	(140.55-307.83)	2.10*
Diltiazem (25 mg/kg sc) +Standard antidotes	30	120.75	(63.35-230.62)	0
	0	0	(129.66-314.76)	2.04*
Nicardipin (10 mg/kg sc) +Standard antidotes	30	121.75	(91.06-163.32)	0
	0	0	(113.82-171.24)	1.41*

Standard antidotes: atropine (10 mg/kg *im*) + oxime HI-6 (10 mg/kg *im*) + diazepam (2.5 mg/kg *im*) were always given immediately after soman (*sc*)

$$PI = \frac{\text{LD-50 of soman in treated animals}}{\text{LD-50 of soman in the control}}$$; *p<0.05 *vs* the control

97. BIOCHEMICAL AND HISTOLOGICAL ALTERATIONS IN RATS SUBACUTELY POISONED WITH T-2 TOXIN

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Aim of this experiment was to ascertain which biochemical and morphological lesions could be produced in rats after subacute poisoning with T-2 toxin, a trichothecene Fusarium species mycotoxin. Males Wistar rats weighing 220-250 g were treated with daily doses of 0.1 LD_{50} of T-2 toxin sc during 28 days. Every week a group of six rats was sacrificed, in order to obtain blood for biochemical analyses and livers, hearts and kidneys for histological preparations.

The activities of all the enzymes monitored were increased significantly in comparison with the control after 7, 14 and 21 days, while after 28 days their activities were significantly decreased. The greatest values of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum activities were registered on day 14 of the experiments, when they were increased by 1.48 and 1.5 times, respectively. Highest serum activities of lactate dehydrogenase (LDH) and creatine kinase (CK) were found on day 21 of the study, when 3.76- and 3.93-fold increase in comparison with the control could be seen, respectively.

Hepatic injury varied from the intracellular oedema, vacuolar and hydrops degeneration to cell necrobiosis and necrotic alterations with primarily centrolobular localization. Parenchymatous and hyaline degeneration of the heart muscle and small necrotic foci and mononuclear cellular infiltration was found. In kidneys, dystrophy and hemorrhagic necroses were established.

These data suggest that T-2 toxin induced lesions of parenchymatous organs and enzyme leakage from them during the first three weeks of the experiment, followed by a deprivation of the enzyme cellular stocks.

Keywords: T-toxin, mycotoxins, enzymes, lesions, rats

99. MEMANTINE TREATMENT IMPROVES ANTIDOTAL EFFICACY OF ATROPINE, HI-6 AND DIAZEPAM IN RATS POISONED WITH SOMAN

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INTRODUCTION

Memantine (1-amino-3,5-dimethyl adamantane) hydrochloride is an adamantane derivative registered as Akatinol® by Merz+Co, GmbH & Co., Frankfurt/M, FR Germany. It has been widely used in many European countries for treatment of various neurological and psychiatric disorders, including Parkinson's and Alzheimer's disease (1, 2). However, since 1987 Dr. Ramesh C. Gupta and his associates from Hopkinsville, Kentucky, USA, have published more than a dozen papers on prophylactic use of memantine alone or with atropine, in rats poisoned with various carbamate insecticides carbofurane (3-7) and aldicarb (8-10), organophosphate insecticide methylparathion (11, 12) and nerve agents tabun, sarin, soman, VX and DFP (13-16). According to these research data, memantine efficiently protects acetylcholinesterase (EC 3.1.1.7; AChE) from inhibition and alleviates tremor, convulsions and skeletal muscle lesions induced by sublethal doses of the cholinesterase inhibitors mentioned (14).

Previous experiments performed in our laboratory confirmed the prophylactic efficacy of memantine against toxicity of various carbamates and organophosphates in rats (17, 18) and mice (19-21). The prophylactic efficacy of memantine in rats poisoned with soman was superior over the potency of classical prophylactic antidotes physostigmine and pyridostigmine (22, 23). In these experiments it was clearly shown that pre-treatment of rats with 18, 36 and 72 mg/kg sc of memantine increases atropine/HI-6 protective index (PI) against soman 2-4 times (24).

However, its cardiovascular and especially its behavioural adverse effects limit the prophylactic use of memantine (25-27). Having in mind that the life-threatening intoxication put the adverse effects of the antidote behind its therapeutic potential, the aim of this investigation was to ascertain the antidotal efficacy of these doses of memantine when administered after soman, i.e. along with standard antidotes.

MATERIALS AND METHODS

Animals. The experiments have been performed with male Wistar rats, weighing 180-250 g, with free access to food and tap water.

Chemicals. Memantine hydrochloride (Akatinol®) was kindly supplied by Dr. Guenter Quack of Merz + Co. GmbH, Frankfurt am Main, FR Germany. Oxime HI-6 (1-(2-hydroxyimminomethylpyridinium)-1-(4-carboxyamidopyridinium) dimethylether dichloride monohydrate) and soman (1,2,2-trimethylpropylmethilphosphonofluoridate) were synthesised in the Military Technical Institute, Belgrade, FR Yugoslavia. Atropine and diazepam were purchased from commercial sources. Memantine and soman were administered subcutaneously (sc), while atropine, HI-6 and diazepam were injected intramuscularly (im).

Procedures. For the purpose of the protective experiments, groups of eight rats each were poisoned with increasing doses of soman and immediately thereafter treated with one of three doses of memantine (18, 36 or 72 mg/kg), and/or atropine 10 mg/kg, HI-6 50 mg/kg and diazepam 2.5 mg/kg. These antidotes were administered either as single regimens or in combinations. After the 24-hour survival registration and calculation of the median lethal dose (LD_{50}) (28), protective indices were calculated as ratios of LD_{50} values in treated and untreated animals, respectively (29).

For the purposes of biochemical experiments, rats were poisoned with maximum sub-lethal dose of soman (0.75 LD_{50}), and immediately thereafter treated with HI-6 50 mg alone, memantine (18, 36 or 72 mg/kg), or with the combination of the oxime and one of these three doses of memantine. One hour after soman administration, animals were sacrificed and AChE activity determined in the homogenates of their brains and diaphragms by Ellman's spectrophotometric method (30). The results were expressed as percentages of the enzyme activity in rats treated with saline only. All the comparisons were made with the AChE activity in rats treated only with soman.

RESULTS

Protective experiments. Among the classical antidotes, only HI-6 significantly protected rats from soman, increasing its LD_{50} by 55%. Atropine + HI-6 was the only dual combination of the classical antidotes, which yielded a significant increase in PI to 2.14. Addition of diazepam only slightly increased that PI to 2.69. Memantine 36 mg/kg alone also assured a significant PI of 1.36. All three used doses of memantine potentiated PIs of all the antidotal regimens investigated. The greatest increases in PI were registered after atropine + HI-6, from 2.14 to 4.70, and atropine + HI-6 + diazepam treatment, from 2.69 to 5.03 (Figure 1).

Biochemical experiments. Soman per se decreased brain AChE activity to 53%. While treatment of rats with HI-6 did not result in higher brain AChE activity in comparison with the untreated but poisoned animals (51% vs. 53%), all three doses of memantine alone significantly protected brain AChE, preserving thus 69-74% of its control activity. Combination of two greater doses of memantine and HI-6 assured the maintenance of enzyme activities close to the control levels (89-97%) (Figure 2).

The activity of AChE in the diaphragms of the rats poisoned with soman and left untreated was decreased to only 30% of its control values. Administration of HI-6 alone doubled it to 60%. All three doses of memantine, administered as monotherapy, assured similar or even higher enzyme activity (56-70%). Combinations of these three doses of memantine with HI-6 resulted in the dose-dependant increase of the diaphragmal AChE activity to 51%, 88% and 107% of the control (Figure 3).

DISCUSSION

HI-6 is, along with HLö-7, the most effective oxime in the treatment of experimental soman intoxication (31-34). These results confirm our previous finding that the administration of the oxime HI-6 monotherapy can significantly prevent death in soman-poisoned rats (18), although no reactivation of brain AChE could be seen. Therefore, its therapeutic effect should be ascribed to the combination of the reactivation of the diaphragmal AChE and the so-called "direct pharmacological effects" of the oxime (35-38).

Addition of atropine, an antagonist of the muscarinic receptors in brain, heart and bronchi, further increased PI via blockade of the effects of the excess of acetylcholine in these vital organs (39), which frequently results in a clear therapeutic synergism (40). Although it is well known that diazepam acts as an anticonvulsive drug counteracting soman-induced seizures (41, 42), it did not in this experiment significantly further increase the PI of atropine + HI-6 combination. One of the reasons could be the erratic absorption of diazepam emulsion from the skeletal muscle (43); the reason why attempts have been made in order to replace diazepam with hydrosoluble benzodiazepine midazolam (44, 45).

The results presented here and preliminary reported earlier (46) clearly show that memantine protects rats from death caused by soman intoxication and potentiates the antidotal effects of atropine, HI-6 and diazepam and their combinations. Also, administration of memantine leads to a significant preservation of AChE activities both in the brains and in the diaphragms of the poisoned animals. Moreover, it strongly potentiates reactivating effects of HI-6 in the diaphragm and even makes it significant in the brain, where HI-6 alone exerted no biochemical effect.

These results confirm Dr. Gupta's and our previous findings that memantine prevents or delays AChE inhibition by soman both *in vitro* (16, 18, 47) and *in vivo* (12, 13, 48). Since memantine lacks the oxime group, it is hard to believe that it can reactivate already inhibited enzyme, like HI-6 does. A more plausible hypothesis concerning the exact biochemical mechanism of memantine interaction with AChE could be allosteric modulation of the active centre of the enzyme, which in turn could lead to its hindrance and unavailability to soman. Another proposed biochemical mechanism could be interaction with soman detoxification by plasma carboxylesterases (EC 3.1.1.1) (49).

The biochemical nature of the antidotal activity of memantine in the peripheral tissues, i.e. respiratory muscles was corroborated by a finding that memantine could alleviate soman-induced deficit in the contractility of the rat phrenic nerve-diaphragm preparation in situ, but failed to induce a similar effect on the neuromuscular deficit induced by decamethonium, a direct nicotinic receptor agonist (50). This experiment ruled out the hypothesis that memantine could exert its peripheral antidotal effect via curariform blockade of the neuromuscular nicotinic receptors (14).

Memantine is also well-known as an anticonvulsive agent, the action of which is ascribed to its neuroprotective effect (51). Memantine is, along with ketamine and dizocilpine (MK-801), one of the drugs that block the action of the excitotoxic amino acid glutamate on the N-methyl-D-aspartate (NMDA) receptors in the central nervous system (14, 15, 52, 53). While soman-induced convulsions are undoubtedly initiated via cholinergic mechanism (54), they are supposedly propagated and the neuronal damage is produced by glutamatergic pathway. This pathway involves not only NMDA, but also AMPA receptors (55). Although this study was not aimed to investigate this mechanism

of antidotal activity of memantine, there is a considerable body of evidence that this mechanism could be of equal value as the biochemical one described above.

CONCLUSIONS

It is concluded that memantine treatment represents an efficient adjunct to standard antidotes against soman intoxication in rats, which is at least partly a consequence of protection of acetylcholinesterase activity in vital tissues. Memantine's anticonvulsive action, however, cannot be ruled out as a one of the antidotal mechanisms.

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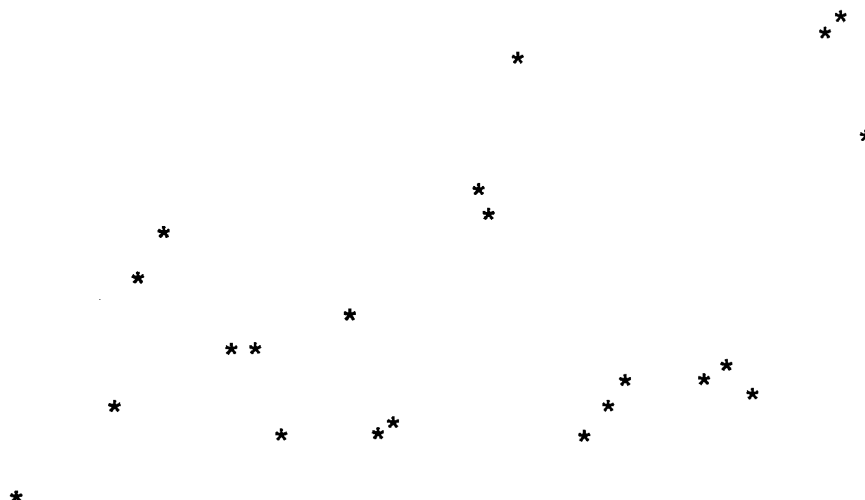
KEYWORDS
Memantine; soman; atropine; HI-6, diazepam

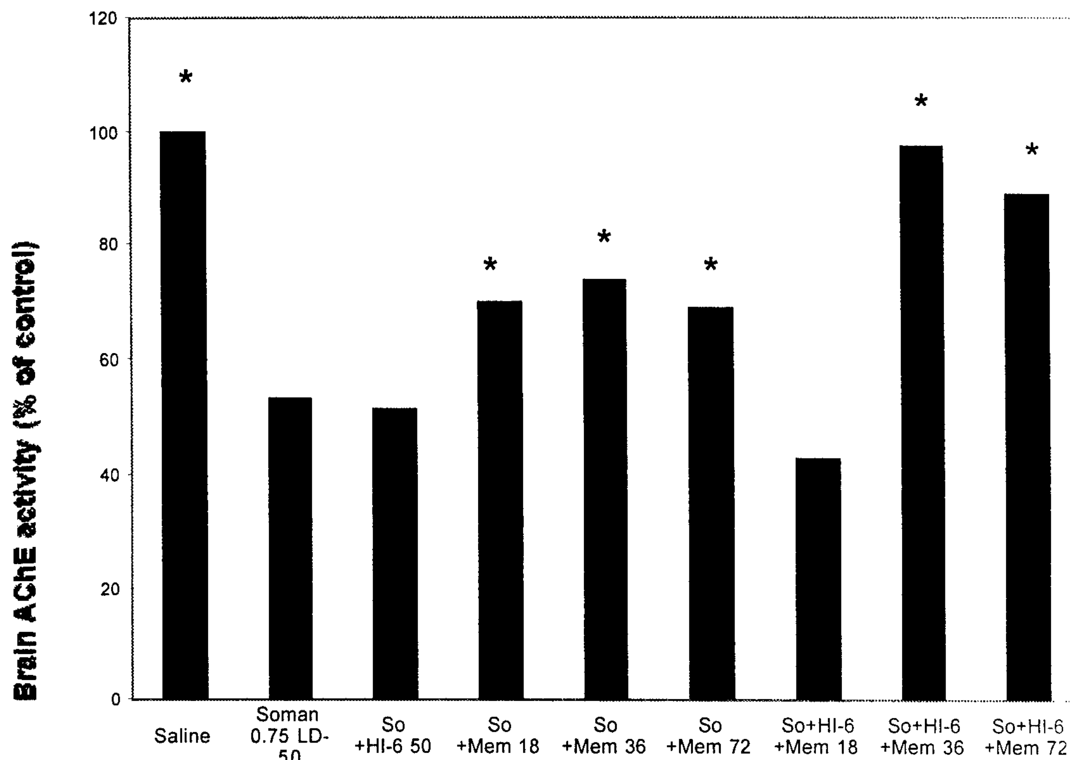
FIGURES AND TABLES

Figure 1 - Protective indices of various antidotal combinations with memantine (18, 36 and 72 mg/kg sc) in rats acutely poisoned with soman sc



* $p < 0.05$, in comparison with animals treated without memantine

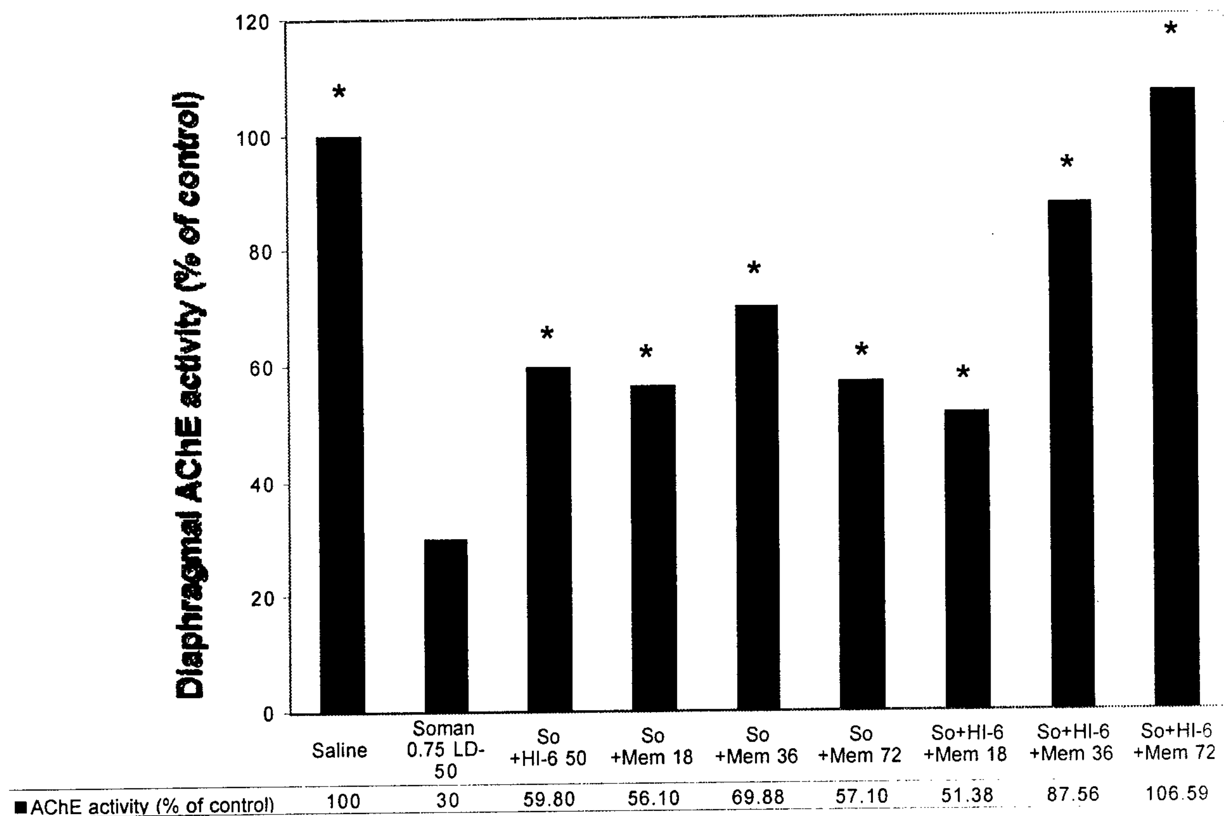
Figure 2 - Influence of HI-6 (50 mg/kg i. m.) and memantine treatment (18, 36 and 72 mg/kg sc) on brain acetylcholinesterase (AChE) activity in rats acutely poisoned sc with 0.75 LD-50 of soman.



	Saline	Soman 0.75 LD-50	So +HI-6 50	So +Mem 18	So +Mem 36	So +Mem 72	So+HI-6 +Mem 18	So+HI-6 +Mem 36	So+HI-6 +Mem 72
■ AChE activity (% of control)	100	53.18	51.18	69.69	73.59	68.82	42.72	97.37	88.95

* $p < 0.05$, in comparison with animals treated with soman only.

Figure 3 - Influence of HI-6 (50 mg/kg im) and memantine treatment (18, 36 and 72 mg/kg sc) on diaphragmal acetylcholinesterase (AChE) activity in rats acutely poisoned sc with 0.75 LD-50 of soman.



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MATERIALS AND METHODS

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CONCLUSIONS

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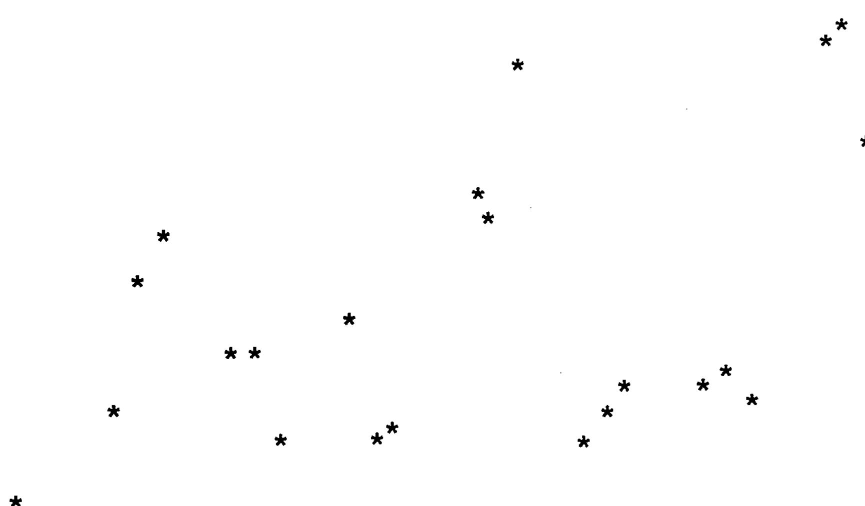
KEYWORDS
Memantine; soman; atropine; HI-6, diazepam

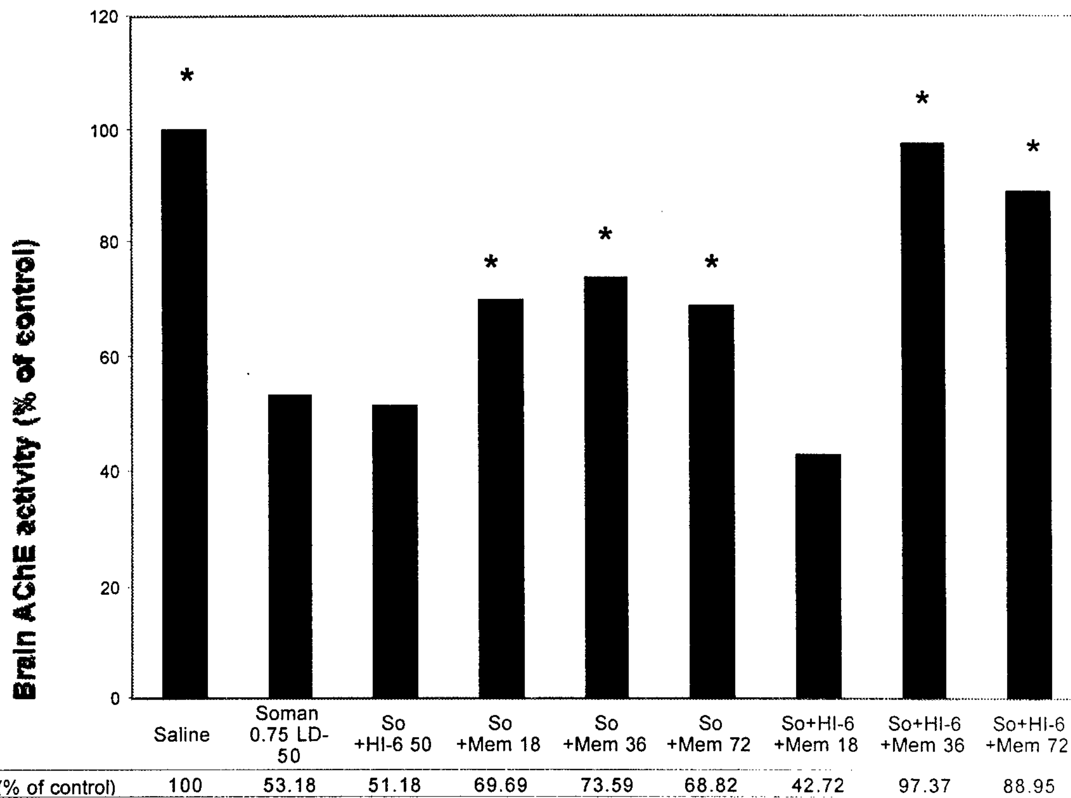
FIGURES AND TABLES

Figure 1 - Protective indices of various antidotal combinations with memantine (18, 36 and 72 mg/kg sc) in rats acutely poisoned with soman sc



* $p < 0.05$, in comparison with animals treated without memantine

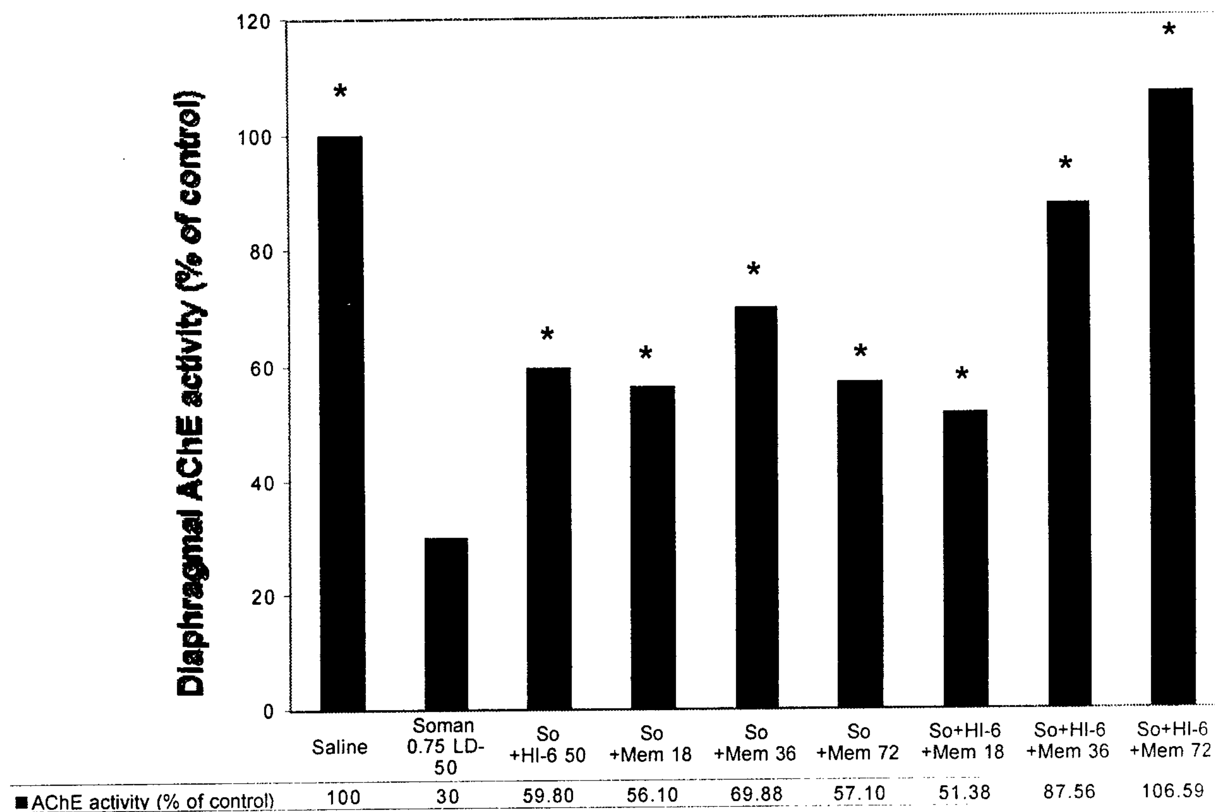
Figure 2 - Influence of HI-6 (50 mg/kg i. m.) and memantine treatment (18, 36 and 72 mg/kg sc) on brain acetylcholinesterase (AChE) activity in rats acutely poisoned sc with 0.75 LD-50 of soman.



	Saline	Soman 0.75 LD-50	So +HI-6 50	So +Mem 18	So +Mem 36	So +Mem 72	So+HI-6 +Mem 18	So+HI-6 +Mem 36	So+HI-6 +Mem 72
■AChE activity (% of control)	100	53.18	51.18	69.69	73.59	68.82	42.72	97.37	88.95

* $p < 0.05$, in comparison with animals treated with soman only.

Figure 3 - Influence of HI-6 (50 mg/kg im) and memantine treatment (18, 36 and 72 mg/kg sc) on diaphragmal acetylcholinesterase (AChE) activity in rats acutely poisoned sc with 0.75 LD-50 of soman.



	Saline	Soman 0.75 LD-50	So +HI-6 50	So +Mem 18	So +Mem 36	So +Mem 72	So+HI-6 +Mem 18	So+HI-6 +Mem 36	So+HI-6 +Mem 72
■ AChE activity (% of control)	100	30	59.80	56.10	69.88	57.10	51.38	87.56	106.59

* $p < 0.05$, in comparison with animals treated with soman only.