

# Health Risks and Preventive Research Strategy for Deployed U.S. Forces from Toxicological Interactions Among Potentially Harmful Agents

by Raymond S. H. Yang<sup>1</sup>

## ABSTRACT

*The goal of this paper is to recommend to the Department of Defense (DOD) a preventive research strategy for deployed U.S. forces to prevent future illness from toxicological interactions from potentially harmful agents. By doing so, it is implicit that potential health risks exist in deployments because of possible exposures to multiple chemicals, drugs, and biologics under stressful environmental and occupational conditions similar to those in the Persian Gulf War. This conclusion was reached based on the author's knowledge of toxicological interactions among chemicals and other agents and his assessment of the available literature information to date. It should be emphasized that this is not an effort to provide an exhaustive review of the field of toxicological interactions of chemical mixtures and other stressors. In fact, some of the areas are so new that the knowledge base is embryonic at best. DOD, through the National Research Council (NRC), seeks expert advice because of the limited information in the area of adverse health effects resulting from multiple stressors, including exposure to chemical mixtures, drug mixtures, vaccine mixtures, and physical and biological agents under highly stressful and hazardous environmental and occupational conditions. Furthermore, psychological stress undoubtedly plays a role in the potential development of such adverse health effects. There is probably no one individual or any group of individuals who knows the answers to such complex situations. Therefore, the author's opinions are, in some cases, based on educated guesses.*

*Given the principal goal stated above, this paper:*

- 1. Discusses the current thinking on toxicological interactions at low-exposure doses, principally to chemicals. However, known and potential toxicological interactions involving biological and physical agents, as well as stressful environmental conditions, are also discussed.*
- 2. Provides an assessment based on experimental toxicological studies of the effects of agents known to be present in the Persian Gulf War. The concerns about the surprising toxicological interac-*

---

<sup>1</sup>Center for Environmental Toxicology and Technology, Departments of Environmental Health, Colorado State University, Foothills Campus, Ft. Collins, CO, 80523-1680.

tions discovered after the Persian Gulf War are discussed. These new discoveries offer potential explanations for the Gulf War Syndrome.

3. Illustrates the importance of the mechanistic understanding of the disease process through research by summarizing some of the studies reported in the literature, which offers a possible explanation for the neurotoxicities of the Gulf War Syndrome.

4. Looks into the rediscovered area of hormesis, as well as the little-known area of multiple stressors. Their potential roles in the field of toxicological interactions are discussed.

5. Explains genetic polymorphism as a basis for sensitive populations. A specific example in experimental toxicology involving multiple stressors is given as an illustration.

6. Offers a preventive research strategy to DOD to avoid possible future Gulf War Illnesses in deployed forces. The rationale, significance, and how-to's for such a preventive research strategy are given in detail.

7. Discusses the ongoing and possible future development of predictive tools for toxicological interactions among chemicals, drugs, biologics, physical and biological agents, and other multiple stressors. Philosophical issues and future perspectives in the context of the present task are also discussed.

## INTRODUCTION

A common definition of an "expert" is one who knows more and more about less and less. Implicitly, this suggests that an expert is very, very focused and is likely to be knowledgeable in a very narrow field. If this is true, what happens when an extremely broad and complex situation like Gulf War Syndrome arises? Do we find many experts in their own respectively focused fields and hope to put the pieces of the puzzle together to form the mosaic? Who is going to see the mosaic? The experts collectively? or a wise old man or woman who knows it all?

The National Research Council (NRC), under the sponsorship of the Department of Defense (DOD), initiated in January 1998 a project on Strategies to Protect the Health of Deployed U. S. Forces. The goal and central theme are succinctly expressed as follows:

The project will advise DOD on a long-term strategy for protecting the health of our nation's military personnel when deployed to unfamiliar environments. Drawing on the lessons of the Persian Gulf War (PGW) and subsequent deployments, it will advise the DOD with regard to a strategy for managing the health and exposure issues faced during deployments to unfamiliar environments; these include infectious agents, vaccines, drug interactions, and stress. It also will include adverse reactions to chemical or biological warfare agents and other substances. In addition, the project will deal with the problem of limited and variable data in the PGW context; and in the development of a prospective strategy for improved handling of health and exposure issues in future deployments. The project will also assess the DOD's response to the recommendations of other expert reports, such as those of the Defense Science Board, the Presidential Advisory Committee on Gulf War Veterans Illnesses, the Institute of Medicine, etc. These tasks would be accomplished with a good understanding of DOD's need to make trade-offs or set acceptable levels of risk.

The broad charge was translated into the following four tasks:

Task 2.1: An analytical framework for assessing the risks to deployed forces from a variety of medical, environmental, and battle-related hazards, including chemical and biological agents (CBA);

Task 2.2: Improved technology and methods for detection and tracking of exposures to these risks;

Task 2.3: Improved technology and methods for physical protection and decontamination, particularly of CBA; and

Task 2.4: Improved medical protection, health consequences management and treatment, and medical record keeping.

I was approached by the NRC as an “expert” to write one of the six commissioned papers for Task 2.1. The word “expert” was placed in quotation marks because of the implication of narrowness discussed above. In the research work done in our laboratory on toxicological interactions of chemical mixtures, it is interdisciplinary team work in its broadest sense. As discussed later, this team is presently collaborating with petroleum-chemical engineers who have the vision of using a recently advanced computer-modeling technique, structure-oriented lumping (SOL), in biomedical research. From that perspective, I would have much preferred that the NRC approached me as a scientist with a vision, not an expert. If possible, I would like to strive for being the “wise old man” who can see the mosaic. It is with this perspective that I embarked upon the writing of this paper.

### TOXICOLOGICAL INTERACTIONS AT LOW DOSES

What is a “toxicological interaction”? and what is a “low dose”? These two terms must be defined and clarified at the outset. The many definitions of toxicological interaction tend to cause confusion. For this paper, we will stick to a simple definition and an updated one. The simple definition of toxicological interaction is “any toxicological consequence deviating from additivity.” The updated definition, which incorporates current thinking about multiple stressors into an earlier version (Lindschmidt and Witschi 1990; Yang 1997), is: “Toxicological interaction is the combination of two or more chemicals, biological agents or disease vectors, physical agents, or stressful environmental conditions that results in a qualitatively or quantitatively altered biological response relative to that predicted from the action of a single chemical, agent, or stressor. The interaction of the chemicals, biological, physical agents, or stressful conditions might be simultaneous or sequential and the biological response might be increased or decreased.”

Until very recently, “low dose” in toxicology has been an abstract entity. It usually implies anything from no observable effects to sublethal effects. In September 1998, a U.S. General Accounting Office (GAO) report to the Congress on *Chemical Weapons. DOD Does Not Have a Strategy to Address Low-Level Exposures* summarized the variety of definitions of low-level exposure provided by DOD officials (GAO 1998). Among these definitions is a quantitative one—0.2 LD<sub>50</sub>. Although one might argue that the toxicological manifestation of 0.2 LD<sub>50</sub> might range from no effects to frank toxicological effects depending on the steepness of the dose-response curve, it is indeed the first quantitative expression of low dose that I have ever seen. Because this paper is to provide insight into health risks and a preventive research strategy for future deployed U.S. forces from toxicological interactions among potentially harmful agents, low dose is defined here as 0.2 LD<sub>50</sub> or lower for any given chemical, drug, biological or physical agent. It should be noted that 0.2 LD<sub>50</sub> might occur from a near zero dose to very high doses or concentrations, such as moles per unit weight or volume, depending on the toxicity of the chemical.

#### How common are toxicological interactions?

Toxicological interactions, be they at high or low doses, are more prevalent than is realized. It is more of a problem of our ignorance and lack of attention to this area rather than the lack of existence of such interactions.

In a recent publication, Lazarou et al. (1998) estimated that there were over 2.2 million cases of serious adverse drug reactions (ADRs) in hospital patients in 1994 in the United States, and among these cases 106,000 were fatal. During their hospital stay, the patients in the survey statistics were given an average of eight drugs. Compared with other statistics of causes of death, these investigators indicated that ADRs became the fourth to sixth leading cause of death for that year in the United States.

The Lazarou et al. (1998) study is particularly significant to our task here in the following ways:

1. Although Lazarou et al. (1998) attributed ADRs as the cause of these deaths, and not specifically toxicological interactions, the fact that multiple drugs were given rendered toxicological interactions to be most likely the cause. This suggestion is strengthened by the fact that drug-to-drug interactions are so common that a separate volume of the Physicians Desk Reference is dedicated to drug interactions (PDR 1996). Further, in the relatively limited chemical world of central nervous system (CNS) depressant drugs, more than 200 ADRs were documented to have occurred as a result of the administration of two or more of these drugs more than 20 years ago (Zbinden 1976).

2. So many ADR deaths occurred that ADRs were ranked as the fourth to sixth leading cause of death in the United States. In addition, over 2 million cases of sublethal ADRs were identified in that year's hospital patient population. A logical question to follow is how many other cases of ADRs might have gone undetected, perhaps due to misdiagnosis, as the intrinsic problems of the patients?

3. These patients were in the hospitals where such things as the environmental conditions, nutrition, and medical care are presumably optimal. Therefore, multiple environmental and occupational stressors such as what deployed forces might face were not there. What would be the consequences if these hospital patients were also exposed to stressful environmental conditions?

4. These people are sick and weak and their homeostasis is dysfunctional at best. Therefore, they are a sensitive population to these ADRs.

5. Drugs, for therapeutic purposes, were not likely to be given at 0.2 LD<sub>50</sub> levels. Most likely they were given at lower dosage levels. Thus, this indicates human lethality and other sublethal ADRs at very low-level exposures of multiple drugs.

For very low exposures resulting from environmental contamination, most practicing toxicologists would probably consider that toxicological interactions are unlikely. This is due to the common belief that these concentrations, usually at parts per billion (ppb) levels, are far below the saturation levels for most biological processes, particularly for the detoxifying enzyme systems. Are these common beliefs true? To answer this question, Yang (1994) went through some calculations for 1 ppb chloroform in drinking water due to the chlorination disinfection process. Yang indicated that this level of chloroform means there are still more than 5 quadrillion molecules in 1 liter of water. Using a series of illustrations and arguments, Yang concluded that (1) even at 1-ppb level, there are a huge number of molecules in our body; (2) these molecules are not present alone in the sense of chemical species, they are present along with other xenobiotics; (3) there is a very narrow range (probably less than 3 orders of magnitude) between "no effects" and "effects" in the various toxicity studies; (4) toxicological interactions seems possible, at least theoretically, at low-exposure concentrations; however, the sensitivity of detection might pose a problem. Yang's contention was, in part, supported by some experimental findings, particularly the clear dose-related *in vivo* cytogenetic toxicity in rats treated with an ultra low concentration (ppb levels) of a pesticide-fertilizer mixture (Kligerman et al. 1993), and marked carcinogenic activities in a mixture of very low doses (1/50 of TD<sub>50</sub>) of 40 known carcinogens (Takayama et al. 1989).

Considering carcinogenicity as an endpoint in toxicological interactions, a number of studies were published in the literature on multiple chemical exposures. In one series of studies (Elashoff et al. 1987; Fears et al. 1988, 1989), binary mixtures of 12 known or suspected carcinogens were evaluated for tumorigenicity. These investigators observed synergism, antagonism, and lack of interactions. In a review by Arcos et al. (1988) on binary-combination effects of carcinogens, a total of 976 interactions involving almost 200 carcinogens in 10 chemical classes were uncovered. The predominant target organ was the skin, accounting for nearly 50% of all synergistic combinations. Similarly, Rao et al.

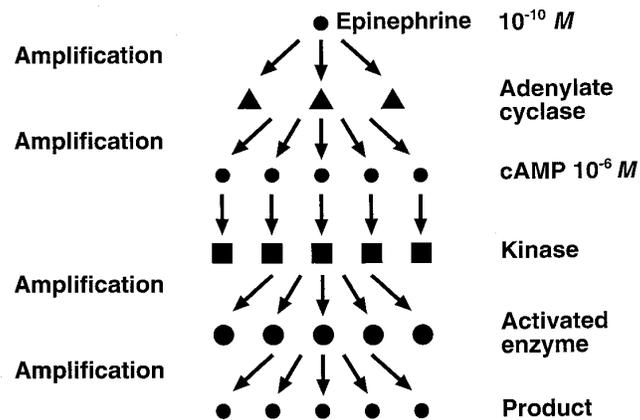


FIGURE 1 An example of cellular transduction and amplification. A single molecule of epinephrine resulted in the synthesis of thousands of cAMP molecules, which, through biological amplification, eventually raised the blood glucose level markedly. (Redrawn from Lodish et al. 1995.)

(1989) examined the literature on 600 tumor promoters or co-carcinogens and found 1,250 interactions involving chemicals from 21 classes.

If toxicological interactions are more prevalent than thought, why are there studies (Feron et al. 1995; Jonker et al. 1996; Cassee et al. 1998; Safe 1998) that support an additivity or less-than-additivity concept at the low-dose region? The answer to this question might be given from a number of different angles. First, some of these low-dose studies, including a number of papers from our laboratory (Pott et al. 1998; 1999; Benjamin et al. 1999; Dean et al. 1999), demonstrated antagonistic interactions, which are one form of toxicological interactions. Second, many of these studies that suggest that at low doses additivity or less than additivity prevails are based on acute or short-term toxic endpoints (Feron et al. 1995; Jonker et al. 1996). Third, because the real concern for environmental contamination is low-dose, long-term effects, acute and short-term toxicity studies cannot and should not be used to extrapolate to hazard identification for chronic toxicities. Finally, if environmental pollutants are active in any process that involves cascading amplification, such as hormonal effects or carcinogenic processes, they might cause toxicological interactions even at very low concentrations. For example, the concentration of epinephrine needed in the blood to stimulate glycogenolysis and release glucose from the liver and muscles can be as low as  $10^{-10} M$ , a stimulus that generates a concentration of more than  $10^{-6} M$  cAMP in the cell. Because three more catalytic steps precede the release of glucose, another  $10^4$  amplification can occur (Figure 1), so that blood glucose levels ultimately increase by as much as 50% (Lodish et al. 1995). If certain environmental pollutants can interfere with this process at the epinephrine level or similar processes with cascading and amplification effects, it is conceivable that disproportional toxicological interactions might happen.

### Chemical-to-Chemical Interactions

In talking about chemical-to-chemical interactions, we are not specifically considering two or more chemicals reacting with one another to create one or more new chemicals before entering the body. Although

this might be a case, we are referring to two or more chemicals causing toxicological consequences within the body deviating from additivity. The discussion in the last section indicates examples of toxicological interactions that may be classified as chemical-to-chemical interactions. Because this paper is not meant to be an exhaustive review of all known examples, more examples can be found in Goldstein et al. 1990; Calabrese 1991a,b; Pollak 1993; EHP 1994; FCT 1996; and Yang 1994, 1997. The additional examples given below are specifically centered around toxicological interactions following low-level exposures.

In the 1980s, the National Toxicology Program (NTP) initiated a large number of studies on the possible toxicological interactions on a number of target organs and systems by low-level exposures (ppm or ppb) to a 25-chemical mixture of groundwater contaminants from hazardous-waste sites, a pesticide-fertilizer mixture, or a herbicide-fertilizer mixture imitating groundwater contaminants in agricultural regions in California and Iowa. In one study, Germolec et al. (1989) reported that suppression of bone marrow stem-cell proliferation, as expressed by the number of colonies formed by the granulocyte-macrophage progenitor cells, as well as suppression of antigen-induced antibody-forming cells (sheep red blood cells) was observed in B6C3F<sub>1</sub> mice following 14-day and 90-day exposures to a drinking water cocktail of 25 groundwater contaminants. In another study, Kligerman et al. (1993) observed *in vivo* cytogenetic changes (increased sister-chromatid exchange) in Fischer 344 rats and B6C3F<sub>1</sub> mice following subchronic exposure to the California pesticide-fertilizer mixture. In this latter study, the six pesticides were at ppb levels, although ammonium nitrate, the only fertilizer given, was at ppm levels. In these studies, no systematic single chemical or submixture studies were conducted because of the complexity involved and limited resources. However, based on the experience and knowledge of the investigators, toxicological effects were deemed unlikely to happen with single-chemical exposure at the dose level tested.

It is interesting to draw parallels between the above two studies and certain findings in humans. For instance, studies conducted in Russia on children with compromised immune and endocrine systems living in different air-polluted, oil-waste regions revealed a dose-response effect of the anthropogenic pollution on T-suppressors as well as stimulation of immunoglobulin synthesis and reduction of phagocytic activity of the neutrophils (Etkina and Etkina 1995). In Greece, agricultural workers exposed to pesticides showed substantial clastogenic effects (chromosomal aberrations) in their lymphocytes without indication of increases in their basal frequency of sister chromatid exchange. Further, it was observed that individuals working exclusively in confined spaces, such as inside the greenhouse, showed higher chromosomal aberration levels than those working in open fields. No significant difference was found between smokers and nonsmokers (Kourakis et al. 1996).

### **Chemical-to-Physical Agent Interactions**

Two examples are given below for chemical-to-physical agent interactions. In three NTP studies on the possible toxicological interactions between a 25-chemical mixture of groundwater contaminants and whole-body irradiation on hematopoiesis (Hong et al. 1991; 1992; 1993), exposure of the chemical mixture to B6C3F<sub>1</sub> mice enhanced the reduced bone marrow stem-cell proliferation resulting from radiation injury following repeated whole-body irradiation at 200 rads. Even 10 weeks after the cessation of chemical-mixture exposure, when all hematological parameters were normal, a residual effect of the chemical mixture might still be demonstrated as lower bone marrow stem-cell counts following irradiation (Hong et al., 1991). Another example of a chemical-to-physical agent interaction involved pesticides and ultraviolet (UV) light. It is commonly known that UV light will degrade hazardous chemicals such as pesticides. However, a study by McCabe and Nowak (1986) demonstrated that some pesticides act synergistically when combined with UV light.

### Chemical-to-Biological Agent Interactions

In the broadest sense, chemical-to-biological interactions include pharmacodynamics of any toxicants because a chemical causes toxicity by interacting with a biological entity, be it an enzyme, nucleic acid, or a protein receptor. Thus, receptor-mediated toxicity such as TCDD-Ah receptor interactions, as well as multistage carcinogenesis from environmental chemicals, should be considered as part of chemical-to-biological interactions. However, for assessing risks to deployed forces, the concern is about the probability of chemical in conjunction with biological agents such as vaccines or disease vectors causing synergistic adverse health consequences. Examples given below are from some of the more recent publications, and they illustrate cases of chemical-to-biological agent interaction in the body leading to changes in pharmacokinetics and pharmacodynamics. Even though these examples mainly involve interactions between trace elements or heavy metals and bacterial or viral agents, a logical question to ask is how prevalent are these interactions among other chemicals and biological agents? These examples certainly suggest the likelihood of chemical-to-biological agent interactions leading to serious toxicities in humans.

Trace elements and heavy metals like copper, zinc, iron, and selenium have a significant influence on the function of the immune system. Srinivas et al. (1988) studied plasma levels of trace elements in 53 patients with acute bacterial and viral infections. They found that plasma concentrations of selenium, iron, and zinc were decreased in patients with bacterial infections (septicaemia, pneumonia, erysipelas, and meningitis). Patients with viral infections showed similar shifts of the trace elements but the changes were not as pronounced. In a series of studies from Sweden, Ljback et al. (1992, 1993, 1994a,b, 1995) and Glynn et al. (1998) reported that:

1. An invading microorganism can increase the intestinal absorption and concomitantly alter the distribution of  $^{109}\text{Cd}$  in Balb/c mice during viral infection (Coxsackie virus B3 [CB3]). Similar studies demonstrated the alteration of distribution of  $^{63}\text{Ni}$  and  $^{14}\text{C}$ -cholesterol in CB3 infected mice.

2. Cadmium exposure for 10 weeks in female Balb/c mice with myocarditis (induced by CB3) resulted in a decreased maturation and mobilization of T and B lymphocytes, but increased humoral immune host responses.

3. A 10-week low-dose (0.002 M) administration of NiCl might contribute to the progression of target organ pathology in infection-induced diseases of an autoimmune or inflammatory character, such as diabetes and myocarditis.

4. The magnitude of inflammatory lesions in the hearts of CB3 infected mice can be affected by the potentially toxic heavy metals—cadmium, nickel, and methyl mercury. The infection is associated with a changed distribution, such as a cadmium accumulation in the spleen and kidneys. New target organs for nickel during the infection were the heart, pancreas, and lungs, in which the inflammatory lesions were present. The increased uptake was correlated with the disturbed function of the immune cells and an increased inflammatory reaction. Nickel and methyl mercury appeared to have a direct effect on immune cells that resulted in changed natural killer-cell activity and decreased mobilization of macrophages, and CD4+ and CD8+ cells into the inflammatory lesions.

Two other studies provide a glimpse of the diversified and intriguing domain of chemical-to-biological agent interactions. Novick et al. (1997) reported that free ionic zinc ( $\text{Zn}^{2+}$ ) in saliva shortens duration and severity of common cold symptoms. They proposed that  $\text{Zn}^{2+}$  forms a complex with proteins of critical nerve endings and interrupts nerve impulses. Further, they suggested that  $\text{Zn}^{2+}$  binds with surface proteins of human rhinovirus (HRV) and blocks docking of HRV on intercellular adhesion molecule-1 on somatic cells, thereby interrupting HRV infection. In the world of plants, chemical-to-

biological interactions might also be present. Ghoshroy et al. (1998) showed that exposure of tobacco plants to nontoxic concentrations of cadmium completely blocked viral disease caused by turnip vein-clearing virus. Cadmium-mediated viral protection was due to inhibition of the spread of the virus from the inoculated into uninoculated leaves.

On a much broader level involving ecological parameters, the study by Porter et al. (1984) summarized later, serves as an example of chemical-to-biological interaction.

### Known Versus Unknown Interactions

The preceding sections have provided many examples of toxicological interactions; they also gave a glimpse of the known toxicological interactions at low doses. However, from the perspective of deployed forces, the possible toxicological interactions unknown to us are surprising and frightening. The examples of toxicological interactions given below, which were not known until the publications appeared several years after the PGW, serve as an illustration.

Two reports from Duke University on the synergistic neurotoxic effects in hens of pyridostigmine bromide (PB), *N,N*-diethyl-*m*-toluamide (DEET), and permethrin or chlorpyrifos in combination (Abou-Donia et al. 1996a,b) had provided some insights to the possible mechanistic basis for the neurotoxic symptoms observed in the veterans afflicted with Gulf War Syndrome. Of course, hens were used in these studies because they are a good animal model for organophosphorus-induced delayed neurotoxicity (OPIDN). PB (prophylactic antinerve gas agent), DEET (insect repellent), permethrin (pyrethroid insecticide), and chlorpyrifos (organophosphorus insecticide) were used by the allied forces in the Persian Gulf War. Although each of these chemicals individually caused little or no toxicity at the doses tested, synergistic neurotoxic interactions were observed when they were given together as binary or ternary mixtures. The synergistic neurotoxic interactions were seen with multiple endpoints, including clinical signs, locomotor dysfunction, histopathological changes, plasma butyrylcholinesterase, brain acetylcholinesterase, and neurotoxicity-target esterase, body-weight changes, survival time, and mortality.

Another surprise involved pyridostigmine and the blood-brain barrier. To many scientists, pyridostigmine was generally considered to be safe and not likely to be involved in the neurotoxic aspects of Gulf War Syndrome because of the common belief that pyridostigmine does not penetrate through the blood-brain barrier. However, in 1996, Friedman et al. in Israel reported that, after mice were subjected to stress (forced swim), the blood-brain barrier literally opened up to pyridostigmine such that the value for a defined effect in 50% of exposed animals ( $ED_{50}$ ) in vivo for brain acetylcholinesterase (AChE) lowered to 1% of that under nonstressed conditions. This finding was further backed up by increased brain levels of *c-fos* oncogene (which might be involved in the induction of AChE transcription) and AChE mRNAs in the stressed and pyridostigmine-treated mice. The results from the above study suggest that peripherally acting drugs such as pyridostigmine administered under stress might reach the brain with an amplification of their action by more than 100-fold and produce serious neurological damage (Friedman et al. 1996; Jamal 1998).

Such heretofore unknown toxicological interactions should raise the consciousness of the scientific community in at least two ways:

1. The findings provided a basis for hypothesizing about what might have happened to the Gulf War veterans with long-term neurotoxic consequences. As Friedman et al. (1996) suggested, the transcriptional responses (i.e., mRNA increases for *c-fos* and AChE) they observed in mice under stress plus pyridostigmine-treatment predict the induction of secondary and tertiary processes that, in turn, might have a number of neurotoxicological consequences. Specifically, Friedman et al. (1996) raised the

possibility that the observed pyridostigmine-induced enhancement of the capacity to produce AChE reflects a potential selective feedback mechanism that would diminish cholinergic overactivation.

2. Clinicians and scientists who hold the view that “what we don’t know doesn’t exist” should be humbled. Indeed, our knowledge in this area is at an embryonic stage.

A third interesting surprise involves the potentiation of PB toxicity in mice by, among others, caffeine. Chaney et al. (1997) reported that male ICR mice received ip injections of either a selected adrenergic drug or caffeine (5 mg/kg), followed 15 minutes later by an intraperitoneal (ip) injection of PB at 1, 2, or 3 mg/kg. Using isobolographic analyses, Chaney et al. (1997) concluded that synergism was demonstrated between PB and several commonly used classes of adrenergic agents and caffeine.

In line with the above discussion, it is also interesting to note the following reports:

1. Hanin (1996) summarized the work on stress and a leaky blood-brain barrier (BBB) and reported that “Acute immobilization stress in rats, cold or isolation exposure in mice and exposure of rats to conditions of acute as well as chronic summer heat, all resulted in increased penetration of the BBB by drugs, neurotransmitters and viruses that are normally excluded”

2. Hubert and Lison (1995) investigated potential muscular damage produced by short-term PB treatment in resting and exercising rats. They showed that, following physical exercise, PB significantly exacerbated the biochemical changes (increased creatine phosphokinase and urinary creatine excretion rate) reflecting a loss of integrity in skeletal muscles.

3. Casale et al. (1993) examined the potential of four carbamate and four organophosphate anti-cholinesterase (anti-ChE) insecticides to inhibit interleukin 2 (IL-2)-dependent proliferation of mouse T cells. Carbaryl and dichlorvos are the most potent agents and the mechanistic basis might be the inhibition of serine hydrolase-dependent immune functions including IL-2 signaling. Casale et al. (1993) proposed that these anti-ChE insecticides may be important immune dysregulators.

4. Rook and Zumla (1997) suggested the symptoms of Gulf War Syndrome might be compatible with the interaction between multiple Thorium (Th) 2-inducing vaccinations and stressful circumstances. They indicated that the mood changes and depression that commonly accompany Gulf War Syndrome can be accounted for by Th 2-mediated disorders.

5. Ben-Nathan et al. (1991) reported that the neurovirulent, noninvasive Sindbis virus strain (SVN), when injected intracerebrally into the mouse’s brain, causes acute encephalitis and death but is unable to pass the blood-brain barrier to invade the brain when injected intraperitoneally. However, when mice were subjected to cold or isolation stress, the blood-brain barrier opened up to the SVN, leading to encephalitis and death.

### **Mechanistic Considerations**

When dealing with toxicological interactions of chemical mixtures, as is the case with single chemicals, mechanistic information is critical, particularly in the development of predictive tools. A summary of mechanistic information is presented in a schematic form (Figure 2) taken from a number of studies related to the possible explanations of neurotoxicities in the PGW veterans (Abou-Donia et al. 1996a,b; Friedman et al. 1996; Haley and Kurt 1997; Jamal 1998).

The top half of the diagram deals with pharmacokinetics and the bottom half depicts pharmacodynamics, what happens inside of the blood-brain barrier. From the top left to right, Figure 2 illustrates three possible exposures and the related pharmacokinetic scenarios in those PGW veterans who might be both members of a sensitive population and who might have had higher levels of exposures to the

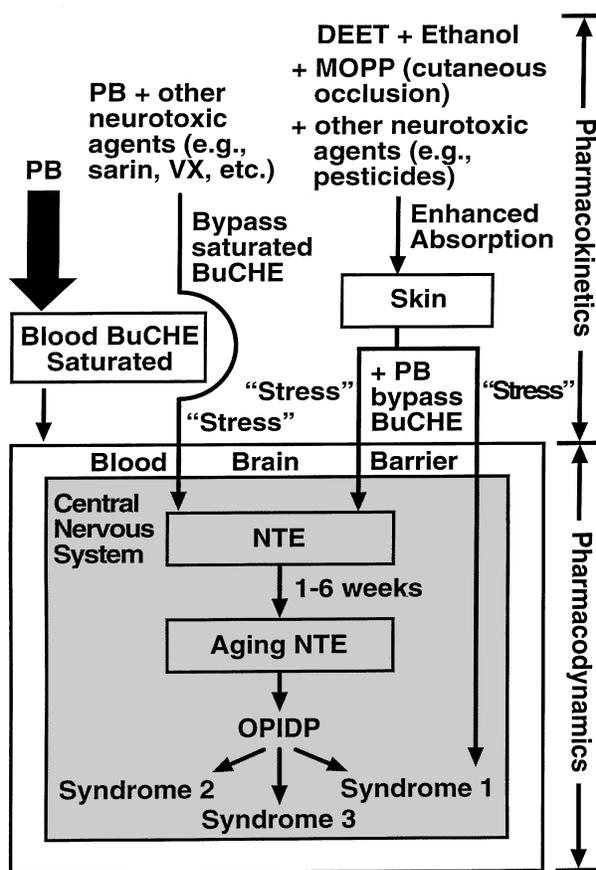


FIGURE 2 A schematic of proposed mechanisms responsible for the neurotoxicities observed in PGW veterans. The synthesis of this schematic was based on mechanistic information proposed in Abou-Donia et al. (1996a,b), Friedman et al. (1996), Haley and Kurt (1997), and Jamal (1998). BuChE = butyrylcholinesterase; DEET = *N,N*-diethyl-*m*-toluamide; MOPP = military operations protective posture; NTE = neuropathy target esterase; OPIDP = organophosphate-induced delayed polyneuropathy; PB = pyridostigmine bromide; VX = nerve gas.

chemicals and drugs implicated as the culprits. First, under normal circumstances, PB upon intake enters into the blood stream where butyrylcholinesterase (BuChE, also called pseudocholinesterase or plasma cholinesterase) binds with PB and renders it unavailable to other possible targets. Thus, BuChE is a body's natural protective mechanism. However, if this protective mechanism is overwhelmed (saturated) because there are also other neurotoxic agents such as sarin and VX in addition to PB then the possibility exists that some of these molecules might bypass BuChE's protective shield and enter the blood-brain barrier. In the third scenario, soldiers had to wear gear called a military operations protective posture (MOPP), which is the equivalent of a "walking Turkish bath" in desert weather conditions. Because the soldier perspires profusely, the MOPP becomes a most efficient occlusion for the body surface. Under such a condition, DEET, ethanol, and other neurotoxic agents such as pesticides might penetrate the skin at much higher rates than normal. Again, if there are PB molecules already bound to BuChE, these other neurotoxic agents might enter the blood-brain barrier without much resistance. Because of stress factors such as not eating or sleeping well, bad food and weather, and fear of death (paralleling the study by Friedman et al. [1996]) the penetration of neurotoxic agents might be further enhanced as indicated in Figure 2. Once these neurotoxic agents are in the CNS, they (mainly organophosphates [OP] and related compounds) react with neuropathy target esterase (NTE, formerly known as neurotoxic esterase). The OP-NTE complex undergoes molecular rearrangement over a 1- to 6-week period to form a byproduct that is axonotoxic and might lead to organophosphate-induced delayed polyneuropathy (OPIDP). The spectrum of clinical effects in OPIDP ranges from permanent

impairment involving distal, wasting peripheral neuropathy and spinal cord spasticity to vague cognitive and behavioral changes (Haley and Kurt 1997). Haley and Kurt categorized the clinical manifestations into three syndromes. Syndrome 1 is impaired cognition, a milder form of neurotoxicity that resembles the chronic effects of pesticide exposure and poisoning. Syndrome 2 is confusion-ataxia, the most disabling form of neurotoxicity in PGW veterans. This syndrome might be the result of toxicological interactions from sublethal exposures to chemical nerve agents in soldiers whose protective pool of BuChE was already diminished by preexposure to PB or pesticides. Syndrome 3 is arthro-myo-neuropathy, which might result from heavy percutaneous absorption of DEET in soldiers whose BuChE pool was already saturated by PB, allowing more of the absorbed DEET to diffuse into the CNS.

It is important to make one point clear here. Whether this summary of mechanistic basis for possible explanation of neurotoxicities in the Gulf War Syndrome is 100% in line with the latest advances in neurotoxicology is not the main issue. The significance is that a group of independent scientists came up with an explanation of the neurotoxicity observed in veterans with Gulf War Syndrome. From my perspective, such a schematic summary of disease processes based on mechanistic information might serve as a conceptual model for the development of an integrated physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) model, as discussed later. With verification using available quantitative data, this model might possess predictive capability for computer simulations of possible future, similar scenarios in deployed U.S. forces. Further, with potential additional information provided by cutting-edge researchers in neurotoxicology or from advances in future studies, the PBPK/PD model might be refined and improved.

### **Hormesis and Potentially Beneficial Interactions**

Hormesis, defined by Stebbing (1982) as the stimulatory effects caused by low levels of toxic agents, was originally developed under different terminologies over a century ago. Its origin, scientific development, historical perspectives, and modern implications have been ably reviewed by a number of contemporary scientists such as Stebbing 1982; Calabrese 1997; Calabrese and Baldwin 1997a,b; Stebbing 1997; Appleby 1998; Bailer and Oris 1998; Gaylor 1998; Johnson and Bruunsgaard 1998; Morré 1998; Morse 1998; Sielken and Stevenson 1998; Teegarden et al. 1998. Dr. Edward J. Calabrese and his colleagues at University of Massachusetts should be credited for the most recent resurgence of scientific interest in hormesis.

The research group headed by Dr. Calabrese searched various computer databases and came up with over 8,000 studies potentially relevant to hormesis. Following a set of criteria related to experimental design, the types of responses and the magnitude and statistical significance of such responses, and the capacity of data replication, Calabrese (1997) and Calabrese and Baldwin (1997a) reported that 500 studies have shown evidence of hormesis to some degree. These investigators summarized their findings as follows.

1. Low-dose stimulatory responses are not restricted to any particular taxonomic group but are observed broadly across the microbial, plant, and animal kingdoms.
2. The types of agents shown to cause hormesis consist of all chemical classes and different types of physical stressors, including various kinds of radiation.
3. Hormesis involves a wide range of biological effects, including growth, longevity, reproduction, disease incidence, and behavioral changes.

To provide an actual example of the hormesis concept, Calabrese (1997) used the differential effects of antibiotics as an illustration. Antibiotics are supposed to kill or prevent bacteria from reproducing.

However, low doses of streptomycin would actually enhance reproduction of certain harmful bacteria even though it kills these bacteria at high doses. In fact, a higher killing capacity of the microbe to the host can be induced by the administration of low doses of streptomycin. This latter scenario is certainly an example of chemical-to-biological interaction in a very negative sense to the host.

Because hormesis is a stimulatory effect at low doses of a toxic agent, it generally has the connotation of being "good." In parallel with the concept of essential metal elements for our growth and homeostasis, hormesis might thus be considered as a form of beneficial effect from otherwise toxic substances at higher doses. Therefore, hormesis is an important consideration here because it occurs at low to very low doses and because, assuming that hormesis might occur with multiple chemicals, including chemical mixtures with interactions, it might provide a basis for the argument that not all interactions are bad.

### **MULTIPLE STRESSORS AND SENSITIVE POPULATIONS**

This section will cover the emerging subject of toxicological interactions (thus possible adverse health effects) that extend beyond chemical challenges to other forms of stress. The subject of sensitive populations as determined by genetic polymorphism will also be considered.

#### **Multiple stressors: What are they? How much do we know? What is the biochemical basis of stress and response?**

Any chemical, physical, or biological insult to the body is a form of stress and can together form multiple stressors. However, in the context of the Gulf War Syndrome, multiple stressors might also include environmental hardship (extreme heat, poor resting conditions, poor food or waste intake, heavy and nonbreathable equipment and clothing, insect or other pests), occupational hazard (dangerous tasks, injuries from work, exposure to fuels, burning oil field, possible nerve gases, radioactive residues), and psychological stress (the threat of death and injuries, fear of exposure to chemical and biological warfare agents, away from home, poor living conditions). Indeed, in their publication, Haley and Kurt (1997) listed 18 suspected wartime causes of illnesses in veterans of the Persian Gulf War. These are chemical warfare agents; environmental pesticides; pesticides in uniforms; pesticides in flea collars; DEET-containing insect repellents; pyridostigmine bromide; ciprofloxacin; chloroquine; multiple immunizations; smoke from oil well fires; fumes from jet fuel in the environment; fumes from burning jet fuel in tents; petroleum in drinking water; depleted uranium in munitions; chemical agent-resistant coating (CARC) paint on vehicles; combat stress; smoking; and alcohol or cocaine use. The reality is probably even more complex than that. Although some of these individual stressors might have been studied and reported in the literature, little or no information is available on the possible combined actions of multiple stressors.

Stress is defined as a state of disharmony, or threatened homeostasis (Chrousos and Gold 1992). If the homeostasis is disrupted due to physical or psychological stress, intricate neural and biochemical events in the brain and in the endocrine and immune systems act jointly to counter the effects of stress and to reestablish homeostasis (Ember 1998). If homeostasis is not reset, debilitating illness results. Biochemically and physiologically, our hypothalamus-pituitary-adrenal gland axis is mobilized under stress and the glucocorticoid hormone cortisol is secreted. The sympathetic nervous system also is activated to release the catecholamines, epinephrine and norepinephrine. Cortisol and the catecholamines are the principal stress hormones.

The process of the body adapting to regain homeostasis is called allostasis. In humans, high allostatic loads (too much stress hormones) can suppress the immune system, decrease bone mineral

density, weaken muscles, promote atherosclerosis (leading to heart disease), hike insulin resistance (leading to diabetes), and accelerate memory loss. Low allostatic load (too little stress hormones) can result in elevated autoimmune and inflammatory responses. Some PGW veterans suffer symptoms consistent with either too much or too little stress hormones (Ember 1998).

### **Environmental Stressors From Different Regions of the World**

The world has extremely diverse weather and geographical conditions, with a wide range of temperature, humidity, and barometric conditions. Associated with these wide range of variables are the different plant, animal, and microbial forms. Any of these almost infinite number of combinations can be an environmental stressor. Because U.S. forces might be deployed anywhere in the world in the future, the studies of some extreme examples of such weather and geographical variables will be wise. This point is taken into consideration later in the proposed preventive research strategy.

### **Anthropogenic Stressors During Wars**

During the Persian Gulf War, oil wells were set on fire, which created pollution in disastrous proportion. The employment of nerve gas was also suspected. In other war scenarios in urban and rural areas, anthropogenic stressors might include setting fires to buildings and equipment, contaminating water and food sources, destroying roads and bridges, decaying human and farm and pet animal corpses, and purposefully destroying chemical plants, releasing highly toxic chemicals. All these could magnify the already existing long list of stressors to the deployed U.S. forces.

### **Synergistic Interaction Stressors and Chemical, Biological, and Physical Agents**

One landmark study that serves as an illustration of synergistic interaction stressors is that of Porter et al. (1984). These investigators evaluated the combined effects of food, water, an immunosuppressant and plant growth regulator (chlorocholine chloride), a virus (Venezuelan equine encephalitis virus), and an environmental contaminant (PCB) on the growth and reproduction of laboratory mice and deer mice. Using a fractional factorial experimental design, they demonstrated interactive effects among the variables tested. For instance, malnourished mice were more sensitive to virus exposure and environmental pollutants. An important interaction was also observed between chlorocholine chloride and water availability. In standard toxicological testing with water ad libitum, chlorocholine chloride enhanced growth of the young. In water stress conditions (80% of normal unlimited consumption), chlorocholine chloride suppressed growth. Porter et al. (1984) concluded that "Interactions of certain 'harmless' chemicals at low levels may prove deleterious than higher doses of 'dangerous' toxicants acting alone."

Specifically regarding to Gulf War Syndrome, other than the proposed mechanisms for neurotoxicity discussed earlier, Rook and Zumla (1997) also postulated the possibility of changes with the clinical manifestation of depression and mood as interactive effects from exposure to multiple Th 2-inducing vaccinations under stressful circumstances.

### **Human Variability: Genetic Polymorphism and Sensitive Population**

The major advances made in the past decades in the area of genetic polymorphism have been in human responses to drugs. One of the greatest causes of interindividual variation of the effects of drugs, and hence of different clinical implications, is genetic variability in drug metabolism (Meyer and Zanger

1997; West et al. 1997). In that sense, genetic polymorphisms are the results of mutations in the genes for the enzymes critical in drug metabolism; the drug-metabolizing enzyme might have an increased or decreased level or be totally absent. Relatively high frequencies of such variant alleles exist in the human population (Meyer and Zanger 1997); for instance, glucose-6-phosphate dehydrogenase (G6PD) deficiency, the most common inherited enzyme-related idiosyncrasy, affects an estimated 400 million people (Eichelbaum and Evert 1996). Another interesting yet alarming feature of genetic polymorphism is the magnitude of the differences. Just as blue and brown eyes can occur in siblings, the interindividual variabilities in drug metabolism might vary by as much as 10-fold to greater than 1000-fold differences (Inaba et al. 1995). Such high magnitudes, if translated by direct-proportionality into acute drug toxicities, would rival any known toxicological interactions.

Based on the fact that genetic polymorphisms have been shown for most drug-metabolizing enzymes (Meyer and Zanger 1997; West et al. 1997), it is conceivable that genetic polymorphism might exist in a wide variety of biological processes. Indeed, a number of reports implicated the association of genetic polymorphism to environmental toxicology and diseases, including cancers (Hirvonen 1995; Ikawa et al. 1995; Rannug et al. 1995; Raunio et al. 1995; Weber 1995; Bartsch and Hietanen 1996; Feigelson et al. 1996; McFadden 1996; Aposhian 1997; Hong and Yang 1997). In one paper, McFadden (1996) speculated on a possible association of several degenerative neurological and immunological diseases with impaired sulfation of phenolic xenobiotics. Rannug et al. (1995), on the other hand, indicated that although CYP2D6 phenotype and genotype have mainly been related to the incidence of lung cancer, the results from 13 different studies showed an absence of any significant correlation between these parameters. This demonstrates that although genetic polymorphism is an exciting area of research with a great deal of vibrant activities, it is probably still in its infancy.

One specific example of genetic polymorphism that has direct bearing here is a study conducted and reported jointly by scientists from Israel, Denmark, and the United States (Loewenstein-Lichtenstein et al. 1995). In this study, the researchers reported that an Israeli soldier, who suffered severe symptoms following pyridostigmine prophylaxis during the Persian Gulf War, was determined to be homozygous for atypical BuChE. Homozygous carriers of atypical BuChE (under 0.04% in Europe but up to 0.6% in certain subpopulations) are known to suffer postanaesthesia apnea and hypersensitivity to the anti-ChE insecticide parathion. This soldier's serum BuChE and recombinant atypical BuChE had far less binding affinity or sensitivity toward PB and other anti-ChEs. These authors concluded that genetic differences among BuChE variants might explain at least partially some of the long-term adverse consequences associated with the collection of symptoms of the Gulf War Syndrome.

### **Normal Physiology Versus Abnormal Physiology**

The abnormal physiology relevant here is the higher permeability of the blood-brain barrier to PB and other neurotoxic agents when an animal is under stress (Friedman et al. 1996; Hanin 1996); muscular damage produced by short-term PB treatment in exercising rats (Hubert and Lison 1995); important immune dysregulation caused by carbamate and OP anti-ChE insecticides (Casale et al. 1993); and sensitivity of individuals who possess genetic polymorphism with respect to xenobiotic metabolism and pharmacokinetics as discussed earlier.

### **Chronic Pain and Fatigue Syndromes**

Chronic pain or fatigue, as clinical features of an amorphous illness, have been described for centuries in the medical literature. Although a variety of terms have been used to describe these

symptoms, the currently preferred terms are Chronic Fatigue Syndrome and fibromyalgia (Clauw and Chrousos 1997). Systemic conditions characterized by chronic pain and fatigue include Chronic Fatigue Syndrome, fibromyalgia, somatoform disorders, and multiple chemical sensitivity (Clauw and Chrousos 1997). Gulf War Syndrome and Sick Building Syndrome share considerable homology with these illnesses.

In a comprehensive review, Clauw and Chrousos (1997) proposed an integrated model for potential pathogenic mechanisms for the symptoms in chronic pain and fatigue syndromes. The salient points are:

“there is a group of individuals who are genetically predisposed to develop the entire spectrum of illness. Susceptible individuals commonly experience a number of organ-specific illnesses before finally progressing to develop the more debilitating systemic conditions later in life. These illnesses may develop indolently or abruptly, and in the latter instances typically follow exposure to a stressor or series of stressors. Once an individual develops this illness, there is evidence of blunting of the human stress response, which may be manifest as: blunting of one or more hypothalamic-pituitary axes, globally increased peripheral and/or visceral nociception, or instability of the autonomic nervous system. These different axes of the stress response can either independently or concurrently function aberrantly, which may in part be responsible for the tremendous heterogeneity of symptoms. In this model, psychiatric illnesses can occur concurrently and significantly modulate disease activity and symptom expression, and vice versa. Finally, in this paradigm, changes in the immune system and in the peripheral tissues are de-emphasized because there are data suggesting that these anomalies occur because of these central alterations in the stress system.”

It should be noted that the symptoms of Chronic Fatigue Syndrome have been described very frequently in patients with Chronic Neuropsychiatric Syndrome of OP compounds (see Jamal 1997).

### **Gulf War Illnesses: Are Gulf War illnesses real? Can they happen again? What can be done?**

In answering the first question, one must try to put all the available pieces of the puzzle together to see what the mosaic tells us. The pieces of the puzzle available to us, in a broad and general sense, are: (1) the potential exists for synergistic toxicological interactions among PB, DEET, permethrin, and chlorpyrifos plus drugs and biologics; (2) under stress, our body might be more susceptible to other insults from chemical, physical, and biological agents; and (3) genetic polymorphism is prevalent in humans, and the magnitude of such manifestations might be very large in extremely sensitive populations. More specific pieces of the puzzle are presented earlier in the sections on Known versus Unknown Interactions and Mechanistic Considerations. My interpretation of the mosaic is that the Gulf War Syndrome is scientifically and biologically plausible. Therefore, I believe that it is real. As to the second question, given the potentially unlimited combinations among environmental and occupational conditions, prophylactic use of drugs, chemicals, and biologics, and the anthropogenic factors for future deployment of U.S. forces, problems similar to the Gulf War Syndrome can happen again.

What can we do about it? First, we must keep an open mind. Aldous Huxley stated, “Facts do not cease to exist because they are ignored.” This paper has reviewed one surprise after another with respect to toxicological interactions from multiple stressors. A good scientist or clinician should never have the attitude that “what I don’t know doesn’t exist.” Second, with the situation DOD is now in, a short-term, stop-gap preventive strategy must be in place to uncover the potential dangers, such that they can be dealt with before our soldiers must face them. Third, a long-term strategy in investing in basic research specifically toward multiple stressor interactions must be implemented. The details are given in the following section.

## PREVENTIVE RESEARCH STRATEGY

Why preventive measures? The short answer is that we want to avoid any future Gulf War Illnesses in deployed forces. The importance, significance, and how-to's are given in the following sections.

DOD has been criticized for not having a strategy to address low-level exposures (GAO 1998). The very fact that DOD has commissioned NRC with the four tasks mentioned at the beginning is an indication of DOD's desire to map out an overall strategy for future deployed forces that goes beyond just low-level exposures. If the recent directive on *Deployment Health Surveillance and Readiness* (JCS 1998) from the Office of the Chairman, The Joint Chiefs of Staff, is any indication, DOD has only pushed forward in epidemiology study-related areas. Although epidemiological studies from past experiences provide useful information, they are generally after the fact, and they suffer from lack of sensitivity and the confounding problems of dealing with chemical mixtures or multiple stressors.

The potential target areas for the U.S. military forces to be deployed are throughout the world; thus, they cover many different geographical regions and their respective weather and environmental conditions. Adding to these conditions is the potential large number of exposure scenarios combining prophylactic drugs, biologics, chemicals, and anthropogenic factors. To avoid or minimize possible future serious health hazards to U.S. military personnel as a result of these combination exposures, preventive measures are critically needed. Because we cannot conduct toxicological experiments on humans, animal and cell-culture models are probably the next best available approach for preventive research.

In formulating plans for carrying out preventive research, it is important to utilize the recent advances in experimental biology and toxicology to assess the health effects of U.S. troops in potential scenarios of multiple exposures of drugs, biologics, and chemicals, as well as possible environmental stresses in different regions of the world. Because of the complexity of the multiple agents and factors involved, the resource-intensive nature of present-day experimental toxicology might only be a short-term solution in terms of preventive research. The long-term strategy should involve the development of predictive capability. In this regard, the integration of more innovative, efficient toxicological methodologies with computer modeling might be the only realistic way to deal with this dilemma, as discussed later.

### **An Integrated Experimental Toxicology Program Assessing Multiple Stressors**

The program suggested below covers both the short-term (from the present to about 5 years) and the long-term (5 years and beyond) objectives. As DOD does not seem to have a strategy for preventive research on potential health effects in future deployed forces, the short-term program should be implemented as soon as possible. The long-term program will take time because it has to mature in parallel to the advances in biomedical research in general. Thus, a relatively small but persistent support might be prudent for the long-term investment.

The short-term program is largely described in a report by the Committee to Study the Interactions of Drugs, Biologics, and Chemicals in U.S. Military Forces, of the Institute of Medicine (IOM 1996). In that report, the following recommendation was made:

“Ideally, for the most complete assessment of the potential interactions of drugs, biologics, and chemicals in the U.S. military forces, a process such as the following should be adopted. Different regions of the world should be characterized according to weather and geographical conditions; ecosystems; abundance of plant, animal, and microbial species; prevalence of diseases; possible anthropogenic pollutants;

and other environmental conditions. Within each region, a list of the potential dangers military personnel might face regarding possible exposures to warfare agents, chemicals, environmental and physical stresses, diseases, pests, prophylactic drugs and biologics, and so on should be compiled and analyzed. Then, under the climatic conditions of each of these regions, animal studies should be carried out to detect at least the four major toxicity categories (i.e., immunotoxicity, developmental and reproductive toxicity, neurotoxicity, and carcinogenicity).

However, to conserve resources and as a starting point, the committee suggests the following prototype experiments with the understanding that more specific scenarios may be incorporated into the experimental design of subsequent studies as needed. At the minimum, the following combination exposure scenarios should be studied for each of the toxicities mentioned above:

1. the complete combination: drugs, biologics, chemicals whose use is anticipated;
2. drugs whose use is anticipated;
3. biologics whose use is anticipated;
4. chemicals to which exposure is anticipated; and
5. controls

The doses of each entity to be used in the animal studies should be the anticipated level of exposure to the soldiers (on a milligram-per-kilogram, millimole-per-kilogram, or units-per-kilogram basis), which would be the baseline study dose, plus two other dose levels (10 times and 100 times this baseline dose). This recommendation may be considered as a first tier screening for possible adverse health hazards. Any toxicological interaction detected within any of the groups should be a warning flag to DOD, and a decision must be made with respect to the risks and benefits involved in using the agents. Beyond this first tier, any additional studies should be on a case-by-case basis guided by the recommendations of an expert panel of investigators.

Some of the conventional toxicity testing protocols may not be applicable in these studies because they are either too expensive and resource-intensive or not sensitive enough with respect to toxic responses, or both. Therefore, there is a need for continuing refinement and improvement of experimental toxicology methodologies by using the latest advances in molecular biology and genetics and in computer sciences. For example, to deal more effectively with interactions, investigators can use and integrate state-of-the-art advances in (1) computational technology; (2) physiologically based pharmacokinetic and pharmacodynamic modeling; (3) model-directed, unconventional, focused, mechanistically based, short-term toxicology studies; and (4) other mathematical and statistical modeling tools.”

To consider further the above approach suggested by the IOM (1996), a number of refinements and modifications may be added:

1. The different regions of the world need not be so many as to add undue burden to the budget of the preventive research program. Depending on the most likely areas U.S. Forces might be deployed, one might simply have extremely hot or extremely cold regions. Within the former, one might consider combining extreme heat with extreme humidity or extreme dryness. These might easily be simulated in laboratory conditions. If elevation might present a potential factor, hyper- or hypobaric conditions can be reproduced in the laboratory as well. In this way, dealing with a large number of environmental factors can be avoided.

2. Even within the first tier there could be priority setting if research funding limitation becomes an issue. For instance, DOD might decide that, as a starter, only one complete set of experiments will be done under the weather conditions of Desert Storm and Desert Shield (for a region in the world where extreme heat, dry, and desert conditions prevail). Of course, in this case, a complete set of experiments should also be conducted under normal laboratory conditions as controls; these control data might be shared with other sets of future experiments in which other regions of the world would be considered.

3. In the IOM (1996) report, a minimum of five exposure scenarios, the complete combination, drugs, biologics, chemicals, and controls (one control group assuming a common vehicle for the groups) was proposed for toxicological interaction studies. However, additional exposure scenarios such as physical agents, disease vectors (as opposed to biologics, which could include only the vaccines applied to the soldiers), or stress factors such as those described by Friedman et al. (1996) might be added to the design as needed.

4. As our cell and molecular biology methodologies advance further, it is conceivable that cell culture systems might be used in the first tier and save the more resource-demanding animal experiments for confirmatory studies.

5. Psychological stress was considered to be an important factor in the development of Gulf War Syndrome. Therefore, in neurotoxicity studies, a subset of experiments should be designed by neuro-behavioral experimental toxicologists in conjunction with psychologists to assess psychological stress as a factor in the toxicological interactions among multiple stressors.

As part of the short-term program, research efforts to learn maximally from the Persian Gulf War should also be encouraged. For instance, Jamal (1998), in his review article on the potential causes and mechanisms of the Gulf War Syndrome, recommended that more multinational studies be done because such an approach could identify various subgroups with different exposure patterns to various risk factors. In particular, Jamal (1998) indicated that the absence of the Gulf War Syndrome in the French troops, who were not exposed to as many of the risk factors that the American and British soldiers experienced, remains crucial to the understanding of the Gulf War Syndrome.

The long-term program should be aimed at the development of predictive capability. The reason is quite obvious. With so many biologics, chemicals, drugs, physical and biological agents, and environmental and anthropogenic factors to consider, it is impossible to keep on doing experiments, particularly with the methods of present-day experimental toxicology. Because toxicology is really the integration of pharmacokinetics (the fate of chemicals or other agents in the body) and pharmacodynamics (the mechanistic interactions of chemicals or other agents in the body), a systematic research effort to investigate pharmacokinetics and pharmacodynamics in representatives of different classes of biologics, chemicals, and drugs, singly and in combination, will provide the database for PBPK/PD modeling or biologically based dose-response (BBDR) modeling. These studies might be conducted in mammals such as the most commonly used laboratory rats and mice for the generation of a database for PBPK/PD modeling. They might also be conducted in cell cultures for the generation of a database for BBDR modeling. In both cases, the latest advances of molecular biology and genetics should be utilized as much as possible. Only through such integration of more innovative, efficient toxicological methodologies, with modern computational technology and the application of engineering concepts to biomedical research, would we have a chance to develop a predictive approach to deal with this extremely complex area.

### **Data Analyses, Interpretation, and Utility for DOD**

For statistical analyses of the experimental outcome from the above-proposed research, the IOM (1996) report has a section that translates the normal statistical jargon into easy-to-understand English. The following passages provide the fundamentals for statistical analyses of toxicological interactions of multiple agents:

“In the simplest case of two agents, each of which produces a single response, one can plot in two dimensions the set of doses ( $x$ ,  $y$ ), where  $x$  is the dose of Agent 1 and  $y$  is the dose of Agent 2 that produces identical responses. The line connecting this set of doses is an isobole, and its graph is an

isobologram (see Figure 1 in Machado and Robinson 1994). Thus, an isobologram is analogous to a topographic map, in which identical responses correspond to identical elevations. In the simple two-agent case, the two-dimensional plot of isobolograms permits a simple, quantitative interpretation: a straight line is indicative of an additive effect of the two agents, that is, no interaction. A convex isobologram is evidence that the response from the combination of the two agents is less than the sum of their responses, which is an antagonistic interaction. A concave isobologram is evidence that the response from the combination of the two agents is greater than the sum of their responses, which is a synergistic interaction.

Combination of more than two agents must be studied in higher-dimensional space, where lines become surfaces and straight lines become planar surfaces. In 1981, Berenbaum quantified and generalized the isobologram to higher dimensions and used it to detect and characterize interactions of a combination of drugs or chemicals, showing that the contours of the constant response of the dose-response surface are planar if the components of the combination have an effect that is additive. In direct analogy to the two-agent case, if the observed response to the combination is statistically greater than that predicted under additivity, it is concluded that a synergistic interaction has taken place. For increasing dose-response relationships, if the observed response to the combination is statistically less than that predicted under additivity, it is concluded that an antagonistic interaction has taken place. If there is no statistical difference between the response predicted under additivity and the response observed upon exposure to the combination, it can be concluded that the components of the combination do not interact. The logic of the approach outlined above was used by Finney (1964), Berenbaum (1985), and Kelly and Rice (1990), among others, to detect and characterize interactions involving combinations of agents.

The real strength of this approach is that relatively few data are required to implement it. Under the assumption of additivity, in particular, the estimated dose-response surface can be calculated from the dose-response curves for the single agents; such data are likely to be available as a result of earlier product development research. One then needs only to collect additional data on the results of exposure to the combination of interest at the specified doses of the constituents.

The required single-agent dose-response data are likely to include multiple control groups, one for each agent under study, especially if these data were collected from several studies. Ideally, such control data can be used to estimate the background rate of response, although an important consideration is their proper inclusion in the analyses. If all of the single-agent control data are collected simultaneously, there should not be any problem combining them. However, when single-agent data are found in the literature or are collected at points in time that are remote to the time of collection of the combination data, then the problem is similar to the historical control problem discussed by Prentice et al. (1992). In extending earlier approaches, Gennings and Carter (1996) used a single parameter for the background (control) rate and developed a methodology that can be used to detect and characterize interactions by incorporating this parameter into the additivity model three different ways: as a fixed-effects parameterization, as a random-effects approach following Prentice et al. (1992), and as an approach involving the use of estimating equations (Liang and Zeger 1986)."

The approach for detecting interactions outlined above is directly applicable to the study of a particular complex mixture of biologics, chemicals, and drugs, as advocated earlier. Let  $B$  represent a given combination of biologics, let  $C$  represent a given combination of chemicals, and let  $D$  represent a given combination of drugs. The complex mixture is represented by  $B+C+D$ . One set of experiments designed to provide data to be analyzed by the methodology described above determines responses to the following sets of exposures:

Control,  $B+C+D$ , 10 ( $B+C+D$ ), 100 ( $B+C+D$ );  
 Control,  $B$ , 10  $B$ , 100  $B$ ;  
 Control,  $C$ , 10  $C$ , 100  $C$ ; and  
 Control,  $D$ , 10  $D$ , 100  $D$ .

The first set of exposure yields the combination agent data, and the next three sets yield the single-agent data for *B*, *C*, and *D*, respectively.”

As for the interpretation and utility of the results, for the purposes of DOD, the most critical ones would be synergistic toxicological interaction. From such findings, one must seriously consider that similar synergistic toxicological interaction might happen in humans. Accordingly, preventive measures, such as modifying the vaccination protocols to fewer numbers, refraining from using prophylactic agents for nonlife threatening problems, and designing better protective masks and suits to minimize environmental harshness and possible chemical and biological warfare agents might be planned in advance.

### **Independent Scientific Board of Counselors**

The establishment of an Independent Scientific Board of Counselors, if done properly, will be essential to the success of DOD's strategy in protecting future deployed forces. This board will review and approve preventive research activities; it will review the results from such research and determine the subsequent course of action. The composition for this board should include, at a minimum, scientific expertise in the areas of neurotoxicology, immunotoxicology, reproductive and developmental toxicology, carcinogenesis, statistics, computer modeling, toxicological interactions, and multiple stressors. Other types of expertise could be added as such needs arise. The members on this board must have an appreciation of the complexity of the problems related to dealing with deployed forces; therefore, they must also have unusually open minds to accommodate risky research and unconventional thinking.

The single most important function for such a board is to deal with science; the science of today and tomorrow, not yesterday. Therefore, it is absolutely essential that the board members should be those who are engaging actively in related research work. The appointment of a board member should never be based solely on political reasons or prestige of position or institution.

### **Existing Approaches for Assessing and Predicting Toxicological Interactions**

Prediction of health effects usually involves some type of mathematical modeling, which ranges from the classical compartmental pharmacokinetic modeling to the currently advanced PBPK/PD modeling and quantitative structure-activity relationship (QSAR) modeling. Some successes, including those from our laboratory, of prediction of pharmacokinetics, pharmacodynamics, and toxicity (including lethality) following exposure to simple chemical mixtures are already evident in the literature (Andersen and Clewell 1983; Purcell et al. 1990; Sato et al. 1990; Thakore et al. 1991; Tardif et al. 1993; 1995; Barton et al. 1995; El-Masri et al. 1996a,b,c; Pelekis and Krishnan 1997; Tardif et al. 1997; Feng et al. 1998; Yang et al. 1999). The toxicological endpoints of prediction, for instance, include an interaction threshold (El-Masri et al. 1996a,b) and acute lethality due to hepatic injuries (El-Masri et al. 1996c; Feng et al. 1998; Yang et al. 1999).

For more complex mixtures, the integration of PBPK/PD, QSAR modeling, and lumping analysis (a modeling tool in the petroleum industry) might formulate a predictive tool for the health effect (Verhaar et al. 1997). The development of predictive capability of toxicities (including carcinogenicity) for chemical mixtures might borrow from the experience of petroleum-chemical engineers. In the 1960s, the application of lumping analysis rendered it possible to predict gasoline production, fairly adequately, based on a few “lumps” rather than the thousands of individual component chemicals of the petroleum (Kuo and Wei 1969; Wei and Kuo 1969). Thus, even though relatively little is known about the

complex mixture of petroleum, a predictive tool was developed and applied from computer modeling. More recent advances and development in lumping analysis incorporated molecular structures and chemical reaction rules, and the end result—structure-oriented lumping (SOL)—is a powerful tool that can be used to simulate the chemical processes going on in a oil refinery (Quann and Jaff 1996; Quann 1998). Coupled with QSAR, SOL can make much more accurate predictions for boiling points, specific gravity, and absolute viscosity for homologous series. Thus, SOL can literally simulate a oil refinery, providing valuable information regarding product yield of fuel gas, gasoline, jet fuel, diesel and heating oil, lubricating oils, asphalt, and coke. The overall achievement of such a modeling approach, therefore, is not only tremendous economic gain but also much more efficient operation for oil refineries and companies.

A reasonable question to ask is, drawing the parallel that a cell is like a chemical plant, should we not be able to utilize a modeling approach, such as the SOL, to develop a predictive tool for health effects from chemical mixtures (Yang et al. 1998)?

### **New Tools: Computer Modeling and Molecular Biology**

Having actively engaged in the research area of the toxicology of chemical mixtures for the last 16 years or so, I have long since become resigned to the fact that the conventional animal toxicological testing methods would not work for chemical mixtures (Yang 1996, 1997; Yang et al. 1998). By adding other multiple stressors such as biologics, physical and biological agents, and environmental stressful conditions, the situation is even more complex. To have a reasonable chance to deal with multiple stressors as a long-term strategy, there is probably no choice but to fully utilize and integrate (1) computational technology; (2) mathematical and statistical modeling; (3) mechanistically based, short-term toxicological studies; and (4) cellular and molecular biology methodologies. To conserve resources and to be able to handle complex experimental designs, an experimental approach for multiple stressors must at least meet the following critical requirements: (1) be relatively simple, short-term, and inexpensive; (2) apply the best science; (3) display an understanding of the mechanisms of toxicity; (4) have broad applicability; and (5) have predictive capability. Using carcinogenicity as an endpoint, a brief description of one possible approach integrating biomedical research with computer modeling follows.

On the biological front, cell-culture systems of human keratinocytes, will be the main thrust for the evaluation of carcinogenic potentials of the selected stressors. There are many phenotypical and genotypical changes accompanying transformation of human keratinocytes; some are early and others are late events. To cover these changes, primary human keratinocytes, which are commercially available, can be used to study the early events (Dlugosz et al. 1995). A number of immortal cell lines such as HaCat (Boukamp et al. 1988), RHEK-1 (Rhim et al. 1985), and NM-1 (Baden et al. 1987) can be used to study the later events. Selected pertinent biomarkers such as TGF $\alpha$ , TGF $\beta$ , *c-myc*, *c-ras*, and p53 might be studied and time-course quantitative information obtained. Such quantitative information might be utilized to calibrate and verify BBDR models.

On the modeling front, the following conceptual BBDR model (Figure 3), which is a published modification (Portier et al. 1990) of the widely known two-stage Moolgavkar-Vinzon-Knodson (MVK) cancer model, can be used.

In this modified four-stage model, the investigators added damaged normal cells and damaged intermediate cells to provide for the two additional stages. Thus, the emphasis for additional cell types is on genetic aberration, such as formation of DNA adducts, single strand breaks, gene amplification, and chromosomal translocation. Similarly, additional stages might be added to the MVK model by

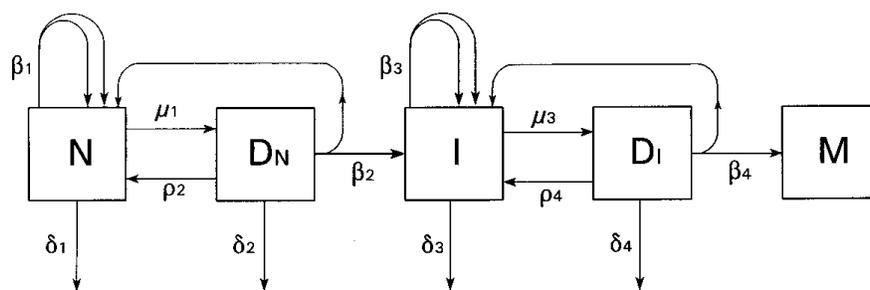


FIGURE 3 A four-stage model of carcinogenesis with clonal expansion and repair. N = normal cells;  $D_N$  = damaged normal cells; I = intermediate cells;  $D_I$  = damaged intermediate cells; M = malignant cells;  $\beta_i$  = birth rate;  $\delta_i$  = death or differentiation rate;  $\mu_i$  = genetic aberration rate;  $\rho_i$  = repair rate. (Reproduced from Portier et al. 1990, with permission.)

inserting cell types with a variety of early or late phenotypical or genotypical changes as long as these changes can be measured quantitatively. A model like this incorporates mechanistic information and, at each cellular stage, the birth, death, and mutation rates are either measurable or can be estimated. Therefore, this is a biologically based model. Because quantitative changes are expected to be related to doses, and the application of this model will certainly involve dose-response simulation and assessment, it is also a dose-response model. When considered in its totality, such a model is a BBDR model.

PBPK/PD models have, up to this point, been associated with whole, multicellular organisms such as mammals, fish, and plants. They describe the interrelationship of physiology, pharmacokinetics, and pharmacodynamics among different organs or lumps of organs or tissues. Although cells have their own physiology and organelles, PBPK/PD modeling has not often been considered with cellular systems. It is possible that a PBPK/PD model can be developed for the cell-culture system when the cell culture vessel is considered as the boundary of the system. Further, as technological advances are made, pharmacokinetics and pharmacodynamics at the subcellular organelle levels might be quantitatively determined. Under those circumstances, the mass balance and transfer, as well as receptor interactions at the subcellular organelle levels, might be described mathematically, and the resulting model, a PBPK/PD model for the cell-culture system, becomes verifiable.

Once the integration of computer modeling and the latest advances of molecular and cell biology becomes a reality, specific toxic responses (e.g., carcinogenic potential, neurotoxicity, developmental toxicity, immunotoxicity) might be assessed at much higher sensitivity levels and be much less expensive. Further development of computer modeling with the incorporation of other technologies, such as SOL, QSAR, and Monte Carlo simulation will enable us to formulate predictive capabilities. As these predictive capabilities are refined and improved through continued research, evaluation of toxicological interactions from multiple stressors might become more and more a matter of computer simulation. Such a developmental strategy will enable us to handle extremely complex situations involving multiple stressors.

### Difficulties in Assessing and Predicting Agent Interactions

Presently, there is some success in predicting toxicological interactions of simple chemical mixtures using the integrated approach of computer modeling and mechanistic toxicology (Andersen and

Clewell 1983; Purcell et al. 1990; Sato et al. 1990; Thakore et al. 1991; Tardif et al. 1993; 1995; Barton et al. 1995; El-Masri et al. 1996a,b,c; Pelekis and Krishnan 1997; Tardif et al. 1997; Feng et al. 1998; Yang et al. 1999). However, there are two major limitations with the results of integrated approaching. One is that the results are relevant only with respect to the specific cases, with little or no extrapolative capability. The other is that all cases dealing with simple chemical mixtures of no more than three chemicals. To overcome these limitations, the next breakthrough must be based on the thorough understanding of the fundamental biology involved in the disruption of homeostasis and the development of a computer model able to handle very complex situations.

### **Opportunities on the Horizon**

Thinking positively and considering that the complexity of issues related to chemical mixtures and multiple stressors are challenges for our intellectual capability, there are many opportunities to be explored and utilized. Nowhere are such opportunities more abundant than in the interdisciplinary research effort that goes beyond the traditional boundaries of fields of studies. For example, can toxicologists look to engineers for help in dealing with the issues of toxicological interactions of chemical mixtures? This author's preliminary experience is that toxicologists can indeed team up with engineers to produce synergistic creativity. The example given below on the potential utilization of SOL is but one such opportunity.

The basic concept of SOL is that any petroleum molecule can be described and represented by a set of 22 structural features or groups called "increments" (Quann and Jaff 1996; Quann 1998); one might consider these increments as the molecular puzzle pieces that form the mosaics of different hydrocarbon molecules. Each of the up to 6,000 hydrocarbon molecules in SOL modeling forms a "vector," a horizontal line to account for the presence or absence of the "increments," which are the 22 columns for the "vectors." This formulates a kind of molecular fingerprint for each of the 6,000 chemicals. The computer can search this matrix of  $22 \times 6,000$  and locate each molecule easily. In addition to this accounting of hydrocarbon molecules, the possible chemical reactions for each of these 6,000 molecules (approximately an order of magnitude greater in numbers; or 60,000 reactions) are also incorporated into the model, according to chemical reaction rules that determine and account for the structural changes (i.e., changes of increments and vectors) of molecules involved in such reactions. Computer programs generate the entire network of chemicals and chemical reactions using sorting procedures to automatically construct the differential rate and energy balance equations for reaction modeling. Expanding further, SOL becomes a molecular-based model for the entire oil refinery by integrating individual-process models.

The idea is that we do not need to know everything, but we have enough information about the overall mosaic to be able to see it. The end result of SOL in petroleum engineering is a much more accurate and powerful predictive capability for both the unknown components and the endpoints of interest such as boiling point, specific gravity, and absolute viscosity of homologous series of petroleum chemicals (Quann and Jaff 1996; Quann 1998).

For the purpose of dealing with the toxicology of chemical mixtures (or to a higher level, the toxicology of multiple stressors), the specific significance of SOL might best be explained in the following way. If SOL can be applied successfully in petroleum engineering to deal with mixtures containing hundreds of thousands of chemical components plus an order of magnitude higher numbers for the chemical processes involved, why can it not be applied to chemicals or chemical mixtures in biological systems? After all, biochemical, cellular, and physiological processes are a collection of chemical and physical processes. There is certainly a tremendous gold mine of knowledge in the literature on physiological and biochemical processes in the cells. Even if our knowledge is incomplete

about some of these processes (like our incomplete knowledge about all the components in petroleum catalytic-cracking processes), computer modeling might be utilized to estimate and predict some of the knowledge for which we do not have enough resources to obtain data empirically. Ultimately, with continuing advances in computer technology and cell biology, it should be possible to model a cell and its related chemical and physical processes for endogenous substances as well as xenobiotics. Such a development might start out with the modeling of as many of the molecular and biochemical pathways as possible for one type of disease process (e.g., cancer). The linkage of these pathways forms a network, and the connection of different networks for different disease processes would eventually provide the mosaic of a homeostatic or disrupted cell that we are looking for. Once this is achieved, molecular-based whole organ, biological system, or even whole body modeling for a human being will not be far behind. Only through this level of modeling the fundamentals of molecular, biochemical, and physiological processes at homeostatic state versus its disruption by chemicals or other stressors is there a real chance of predicting toxicological interactions of various chemical mixtures.

### Philosophical Issues

The discussion of a number of issues might help DOD's effort to formulate strategies to protect the health of deployed U.S. forces.

The first issue is related to the balance between long-term investment versus urgency for answers. The health problems related to the Gulf War Syndrome and the potential adverse health effects under possible conditions for future deployment of U.S. forces cannot be resolved or predicted by quick fixes. The principal reason is that the true answers to those problems are dependent on scientific research and discovery. Because of the complexity and difficulty of the problems, the answers would most likely be uncovered based on painstakingly slow accumulation of knowledge. This is why a long-term strategy in research is necessary. From a different perspective, U.S. forces might need to be deployed anytime, and there must be some information to make health-related decisions. This is where the short-term preventive strategy comes in. As pointed out by the Senate Committee on Veterans' Affairs in the *Report of the Special Investigation Unit on Gulf War Illnesses* in 1998, "The men and women who have served in our nation's military deserve better than what ill Gulf War veterans have experienced."

The second issue relates to the fragmentation and polarization of the scientific community toward certain studies, particularly when they are in the limelight of the news media. Although scientific debates and challenges are healthy and necessary, if they have gone overboard, particularly when scientific opinions are colored by special agendas or emotionalism, the scientific community as a whole becomes the loser. Clinical and epidemiological research on the Gulf War Syndrome yielded results that ranged from no correlation, to some correlation, all the way to strong correlation with Gulf-War-related activities (Gouge et al. 1994; Jamal et al. 1996; Amato et al. 1997; Haley 1997; Haley and Kurt 1997; Haley et al. 1997a,b; Landrigan 1997). Some of these studies led to "war" across the Atlantic Ocean. I have witnessed firsthand part of the controversies surrounding the Abou-Donia et al. (1996a,b) studies and the Haley and Kurt (1997) study. These studies were all published in peer-reviewed journals; thus, at least part of the scientific community approved of these studies. As a scientist without any involvement in any of these studies, I would like to discuss the Abou-Donia et al. (1996a,b) studies objectively to illustrate a point. These two studies will be referred to as the Abou-Donia studies from hereon.

Even though the findings of the Abou-Donia studies were exciting and they might provide some pieces of the puzzle for the mysterious Gulf War Syndrome, a part of the scientific community did not embrace these results. There are probably two principal reasons for such reluctance.

*1. Do animal models, particularly the chicken, reflect what's happening in humans?*

The short answer is yes! From the days of the use of canaries by miners as a preventive measure from occupational hazard, the application of modern experimental biology and toxicology in assessing human health hazard has come a long way. Modern medicine has been built upon the foundation of biomedical research using animal studies. Besides, it is morally and ethically wrong to conduct human toxicological experiments even if there are means to do it. The NRC, in its recent publication on *Science and Judgment in Risk Assessment* (NRC 1994a), indicated the following advantages of animal studies:

- Animals can be used to collect toxicity information on chemicals before marketing, whereas epidemiological data can be collected only after human exposure.
- Animal studies can be controlled, so establishing causation is not in general difficult.
- The quantitative relationship between exposure (or dose) and extent of toxic response can be established.
- The animals and animal tissues can be thoroughly examined by toxicologists and pathologists, so the full range of toxic effects produced by a chemical can be identified.
- The exposure duration and routes can be designed to match those experienced by the human population of concern.

The utility of animal studies in human health hazard assessment might also be underscored by one of the conclusions reached by the Committee to Review the Health Consequences of Service During the Persian Gulf War (IOM 1995) which recommended for immediate action that “appropriate laboratory animal studies of interactions between DEET, PB [pyridostigmine bromide], and permethrin should be conducted.”

However, NRC (1994a) also pointed out that laboratory animals are not human beings. Thus, although animal studies are useful in assessing adverse human health problems, their potential limitations must be recognized. In this context, it should also be pointed out that great variabilities in pharmacological and toxicological responses exist in human populations as well (see discussion of human variability above).

*2. Because the Abou-Donia studies did not establish dose-response relationships, are they applicable to interpreting the Gulf War Syndrome in Gulf War veterans? Are the doses used in these studies relevant?*

For obvious reasons of complexity of design and limitation of resources, both Abou-Donia studies were conducted at single dose levels; thus, a dose-response relationship was not established. This is certainly a weakness of the studies; however, it does not negate the interesting findings with respect to the unanticipated toxicological interactions in the binary and trinary chemical mixtures. These studies certainly suggest the possibility of such toxicological interactions in mammals, including humans. In that sense, scientists and clinicians, in their evaluation of the Gulf War Syndrome in PGW veterans should be well advised to take the above findings into consideration. This was indeed done by some (Haley and Kurt 1997; Jamal 1998).

In their first interaction study (Abou-Donia et al. 1996a), the dose regimen for 5 days/week for 2 months included concentrations of PB in water at 5 mg/kg/day by gavage; DEET neat at 500 mg/kg/day, subcutaneously (sc); and in permethrin in corn oil at kg/day, sc. In their second interaction study (Abou-Donia et al. 1996b), the dose regimen for 5 days/week for 2 months included concentrations of PB in water at 5 kg/day by gavage; DEET neat at 500 mg/kg/day, sc; and chlorpyrifos in corn oil at 10 mg/kg/day, sc. The same doses were given in single-chemical or mixture groups. The researchers have provided very thorough background information on these chemicals and the readers are referred to the original papers for detailed information. The rationale given for the selection of these dose levels and provide my commentary follow.

PB was issued to the military personnel as 30-mg tablet and the recommended dose regimen was one tablet three times daily. That would mean a daily combined dose of 90 mg/person. Assuming an average human body weight of 70 kg, this translates to 1.3 mg/kg/day. In setting their dose, the investigation obviously also considered the human PB therapeutic dose for myasthenia gravis at 200 to 1,400 mg a day (thus equivalent to 2.9 to 20 mg/kg/day), as well as the known acute toxicity in rats (oral LD<sub>50</sub> for PB, 61.6 mg/kg/day). However, comparing the two scenarios strictly on a mg/kg/day basis, the PB dose employed in the studies is about 4 times higher than the intake by the Gulf War veterans. Using 0.2 LD<sub>50</sub> as a definition of low dose as discussed at the beginning of this paper, the PB dose employed by the investigators is about 10 times lower than the 0.2 LD<sub>50</sub> of the rats, estimated at about 12 mg/kg/day. To put it in a different way, assuming that absorption, distribution, metabolism, and elimination are the same between the hen and human, the area under the plasma concentration and time curve (AUC) in the hen would have been 4 times larger than that of the human on a per kilogram body-weight basis. In addition, the human dose was divided into three equal portions daily, whereas the hen dose was given by gavage in one bolus. Therefore, pharmacokinetically, the hen would have had much higher plasma concentration, perhaps 10 times higher, than that of the human. Depending on the window of opportunity for toxicological interactions, this difference can be significant.

DEET is used as an insect repellent against mosquitoes, biting flies, ticks, and other insects. Because there are no reliable estimates of DEET usage among service personnel during the Persian Gulf War, the Abou-Donia studies used a 1986 National Institute for Occupational Safety and Health study of the employees in the Everglades National Park to estimate a reasonably close dosage. In that study (McConnell et al. 1986), the mean weekly estimated dermal application of DEET was 14.6 g, which was equivalent to 42 mg/kg/day for a 70-kg person. It is noteworthy that the top 5% heavy users among the workers in the McConnell et al. (1986) study applied 66.3 g and the top 1% heavy users applied as high as 392.6 g for that weekly period, which was equivalent to applying 189 or 1,122 mg/kg/day, respectively. Given the above information, the selection of neat DEET at a concentration of 500 mg/kg/day, sc, by the Abou-Donia researcher appeared to have been high, even though it is within the upper limit. Considering further that the rat oral LD<sub>50</sub> for DEET is 3,000 mg/kg, using the earlier low-dose definition of 0.2 LD<sub>50</sub>, it could be argued that the 500 mg/kg/day selected for the Abou-Donia studies is still within the low-dose range. Nevertheless, a question that might be on every concerned scientist's mind is "would the Abou-Donia researcher have observed the same toxicological interactions if the DEET dose was set at the estimated mean dose for an Everglades worker at 42 mg/kg/day in their experiments?" An additional point that might have bothered people is the sc injection mode of application in the Abou-Donia studies. Although it is understandable that the feathers are an obstruction for a topical application to chickens, an sc injection certainly bypasses the skin-penetration kinetics and the corn oil solvent would further complicate the absorption kinetics, if applied topically.

For permethrin or chlorpyrifos, both insecticides, the rationale for dose selection was not clear in the Abou-Donia studies. Once again, if using the earlier low-dose definition of 0.2 LD<sub>50</sub>, and given the fact that the chicken oral LD<sub>50</sub> for permethrin and chlorpyrifos are 9,000 mg/kg and 50 mg/kg, respectively, the 500 mg/kg/day for permethrin and 10 mg/kg/day for chlorpyrifos selected for the Abou-Donia studies are both within the low-dose range. Here, the same concerns on sc injection and corn oil as described above would also apply. There is one exposure assessment in the literature (NRC 1994b), however, in which the investigators concluded that, "The average lifetime dermal dose for military personnel from wearing permethrin-impregnated BDUs [body dress uniforms] (permethrin impregnation at a concentration of 0.125 mg/cm<sup>2</sup>) was calculated to be

$6.8 \times 10^{-5}$  mg/kg per day.” If we take this estimate without questioning and compare it with the dose of permethrin Abou-Donia et al. (1996a) applied in this study, there is more than a 7 million-fold difference. Therefore, on the surface, the investigators have seemingly used a totally unrealistically high dose of permethrin in this study. However, upon closer examination, the NRC (1994b) figure of  $6.8 \times 10^{-5}$  mg/kg per day might be unrealistically low. The logic used to reach this suggestion is as follows: The impregnation of BDUs with permethrin is presumably intended to kill insects upon contact. The idea here is that enough selective toxicity favoring humans exists such that a brief contact to the insects will kill them whereas prolonged dermal exposure to human is safe. The soldiers wear BDUs all day long; thus, their exposure to permethrin must be more than the exposure levels for the insects that land and crawl on the BDUs for a brief time. Even assuming the insects, in their brief landing and crawling, get the equivalent exposure ( $6.8 \times 10^{-5}$  mg/g per day) as the soldiers in their BDUs (being sweaty) all day long, I have never known any insecticide that has the lethal effectiveness at 0.000068 mg/g per day. Therefore, something is very wrong in the NRC (1994b) estimate.

Taking the Abou-Donia studies in their totality, and despite their potential weaknesses, my opinion is that they are useful studies, particularly in the context of the Gulf War Syndrome. Given our initial total ignorance of the Gulf War Syndrome and thinking about the tasks ahead of us, I would rather have the knowledge Abou-Donia et al. (1996a,b) contributed in hand in assessing the overall situation. This is particularly true when some of the experimental design weaknesses in the Abou-Donia studies might easily be rectified by follow-up studies in which more resources might be invested to support more thorough dose-response experimental designs.

### FUTURE PERSPECTIVES

The task before DOD is a formidable, but not an impossible, one. It will not be easy; it will require resources; and it will take a long-term, visionary commitment. Any war is a formidable task and DOD has repeatedly demonstrated its ability to live up to the expectation of the nation. Likewise, DOD, with its resources and the available help from the scientific community, should be able to map out a preventive research strategy for the protection of future deployed U.S. forces.

### LIST OF ABBREVIATIONS

BBRD	biologically based dose response
BDU	battle dress uniform
BuCHE	butyrylcholinesterase
DEET	<i>N,N</i> -diethyl- <i>m</i> -toluamide
DOD	Department of Defense
MOPP	military operations protective posture
MVK	Moolgavkar-Venzon-Knudson
NTE	neuropathy target esterase
NTP	National Toxicology Program
OPIDN	organophosphate-induced delayed neurotoxicity
OPIDP	organophosphate-induced delayed polyneuropathy
PBPK/PD	physiologically based pharmacokinetics/pharmacodynamics
PB	pyridostigmine bromide
PCNA	proliferating cell nuclear antigen

QSAR	quantitative structure-activity relationship
SOL	structure-oriented lumping

### ACKNOWLEDGEMENT

The concept development and related research work on chemical mixture toxicology and multiple stressors were supported in part by a Superfund Basic Research Program Project Grant (P42 ES05949) and a research grant (RO1 ES-09655) from the National Institute of Environmental Health Sciences (NIEHS) and a cooperative agreement (#U61/ATU881475) from Agency for Toxic Substances and Disease Registry (ATSDR). Without such generous support for biomedical research, this work could never have been possible.

### REFERENCES

- Abou-Donia, M.B., K.R. Wilmarth, A.A. Abdel-Rahman, K.F. Jensen, F.W. Oehme, and T.L. Kurt. 1996b. Increased neurotoxicity following concurrent exposure to pyridostigmine bromide, DEET, and chlorpyrifos. *Fundam. Appl. Toxicol.* 34:201-222.
- Abou-Donia, M.B., K.R. Wilmarth, K.F. Jensen, F.W. Oehme, and T.L. Kurt. 1996a. Neurotoxicity resulting from coexposure to pyridostigmine bromide, DEET, and permethrin: Implications of Gulf War chemical exposures. *J. Toxicol. Environ. Health* 48:35-56.
- Amato, A.A., A. McVey, C. Cha, E.C. Matthews, C.E. Jackson, R. Kleingunther, L. Worley, E. Cornman, and K. Kagan-Hallet. 1997. Evaluation of neuromuscular symptoms in veterans of the Persian Gulf War. *Neurology* 48:4-12.
- Andersen, M.E. and H.J. Clewell. 1983. Pharmacokinetic interaction of mixtures. Pp. 226-238. in: *Proceedings of the Fourteenth Annual Conference on Environmental Toxicology*, AFAMRL-TR-83-099, Dayton, OH.
- Aposhian, H.V. 1997. Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity. *Ann. Rev. Pharmacol. Toxicol.* 37:397-419.
- Appleby, A.P. 1998. The practical implications of hormetic effects of herbicides on plants. *BELLE Newsletter*. 6(3):23-24.
- Arcos, J.C., Y.T. Woo, and Y.D. Lai. 1988. Database on binary combination effects of chemical carcinogens. *J. Environ. Sci. Health Part C Environ. Carcinog. Rev.* 6(1):1-150.
- Baden, H.P., J. Kubilus, J.C. Kvedar, M.L. Steinberg, and S.R. Wolman. 1987. Isolation and characterization of a spontaneously arising long-lived line of human keratinocytes (NM-1). *In Vitro Cell. Develop. Biol.* 23:205-213.
- Bailer, A.J., and J.T. Oris. 1998. Incorporating hormesis in the routine testing of hazards. *BELLE Newsletter*. 6(3):2-5.
- Barton, H.A., J.R. Creech, G.S. Godin, G.M. Randall, and C.S. Seckel. 1995. Chloroethylene mixtures: Pharmacokinetic modeling and in vitro metabolism of vinyl chloride, trichloroethylene, and trans-1,2-dichloroethylene in rat. *Toxicol. Appl. Pharmacol.* 130:237-247.
- Bartsch, J. and E. Hietanen. 1996. The role of individual susceptibility in cancer burden related to environmental exposure. *Environ. Health Perspect.* 104(Suppl. 3):569-577.
- Ben-Nathan, D., S. Lusting, and H.D. Danenberg. 1991. Stress-induced neuroinvasiveness of a neurovirulent non invasive sindbis virus in cold or isolation subjected mice. *Life Sci.* 48:1493-1500.
- Benjamin S.A., R.S.H. Yang, J.D. Tessari, L.W. Chubb, M.D. Brown, and C.E. Dean. 1999. Lack of preneoplastic foci in rats exposed to a hazardous waste groundwater mixture in a medium-term hepatic initiation/promotion assay. *Toxicology* Submitted for publication.
- Berenbaum, M.C. 1981. Criteria for analyzing interactions between biologically active agents. *Adv. Cancer Res.* 35:269-335.
- Berenbaum, M.C. 1985. The expected effect of a combination of agents: The general solution. *J. Theo. Biol.* 114:413-431.
- Boukamp, P., R.T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, and N.E. Fusenig. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* 106:761-771.
- Calabrese, E.J. 1991a. *Multiple Chemical Interactions*. Chelsea, MI: Lewis. 704 pp.
- Calabrese, E.J. 1991b. *Alcohol Interactions with Drugs and Chemicals*. Chelsea, MI: Lewis. 82 pp.
- Calabrese, E.J. 1997. Hormesis revisited: New insights concerning the biological effects of low-dose exposures to toxins. *Environ. Law Reporter* 27:10526-19532.
- Calabrese, E.J. and L.A. Baldwin. 1997a. The dose determines the stimulation (and poison): Development of a chemical hormesis database. *Int. J. Toxicol.* 16:545-559.

- Calabrese, E.J. and L.A. Baldwin. 1997b. A quantitatively-based methodology for the evaluation of chemical hormesis. *Human Ecol. Risk Assess.* 3:545-554.
- Casale, G.P., J.L. Vennerstrom, S. Bavari, and T. Wang. 1993. Inhibition of interleukin 2 driven proliferation of mouse CTLL cells, by selected carbamate and organophosphate insecticides and congeners of carbaryl. *Immunopharmacol. Immunotoxicol.* 15:199-215.
- Cassee, F.R., J.P. Groten, P.J. van Bladeren, and V.J. Feron. 1998. Toxicological evaluation and risk assessment of chemical mixtures. *Crit. Rev. Toxicol.* 28:73-101.
- Chaney, L.A., R.W. Rockhold, J.R. Mozingo, A.S. Hume, and J.I. Moss. 1997. Potentiation of pyridostigmine bromide toxicity in mice by selected adrenergic agents and caffeine. *Vet. Human Toxicol.* 39:214-219.
- Chrousos, G.P. and P.W. Gold. 1992. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. *JAMA* 267:1244-1252.
- Clauw, D.J., and G.P. Chrousos. 1997. Chronic pain and fatigue syndromes: Overlapping clinical and neuroendocrine features and potential pathogenic mechanisms. *Neuroimmunomodulation* 4:134-153.
- Dean, C.E., Jr., S.A. Benjamin, L.S. Chubb, J.D. Tessari, and R.S.H. Yang. 1999. Interaction of co-planar and non-planar PCBs in promotion of altered hepatic foci. [Abstract]. *The Toxicologist.* 48:235.
- Dlugosz, A.A., A.B. Glick, T. Tennenbaum, W.C. Weinberg, and S.H. Yuspa. 1995. Isolation and utilization of epidermal keratinocytes for oncogene research. *Meth. Enzymol.* 254:3-21.
- EHP. 1994. Environmental Health Perspectives Supplements, Toxicological Evaluation of Chemical Interactions, National Institute of Environmental Health Sciences, Volume 109, Supplement 9, November. 167 pp.
- Eichelbaum, M. and B. Evert. 1996. Influence of pharmacogenetics on drug disposition and response. *Clin. Exp. Pharmacol. Physiol.* 23:983-985.
- Elashoff, R.M., T.R. Fears, and M.A. Schneiderman. 1987. Statistical analysis of a carcinogen mixture experiment. I. Liver carcinogens. *J. Nat. Cancer Inst.* 79:509-526.
- El-Masri, H.A., A.A. Constan, H.S. Ramsdel, and R.S.H. Yang. 1996b. Physiologically based pharmacodynamic modeling of an interaction threshold between trichloroethylene and 1,1-dichloroethylene in Fischer 344 Rats. *Toxicol. Appl. Pharmacol.* 141:124-132.
- El-Masri, H.A., J.D. Tessari and R.S.H. Yang. 1996a. Exploration of an interaction threshold for the joint toxicity of trichloroethylene and 1,1-dichloroethylene: utilization of a PBPK model. *Arch. Toxicol.* 70:527-539.
- El-Masri, H.A., R.S. Thomas, G.R. Sabados, J.K. Phillips, A.A. Constan, S.A. Benjamin, M.E. Andersen, H.M. Mehendale, and R.S.H. Yang. 1996c. Physiologically based pharmacokinetic/pharmacodynamic modeling of the toxicologic interaction between carbon tetrachloride and kepone. *Arch. Toxicol.* 70:704-713.
- Ember, L.R. 1998. Surviving stress. *C&EN* 76(21):12-24.
- Etkina, E.L. and I.A. Etkina. 1995. Chemical mixtures exposure and children's health. *Chemosphere* 31:2463-2474.
- FCT (Food and Chemical Toxicology). 1996. Volume 34, Number 11/12, pp. 1025-1199.
- Fears, T.R., R.M. Elashoff, and M.A. Schneiderman. 1988. The statistical analysis of a carcinogen mixture experiment. II. Carcinogens with different target organs, N-methyl-N-nitro-N-nitrosoguanidine, N-butyl-N-(4-hydroxybutyl)nitrosamine, dipentyl nitrosamine, and nitrilotriacetic acid. *Toxicol. Ind. Health* 4:221-255.
- Fears, T.R., R.M. Elashoff, and M.A. Schneiderman. 1989. The statistical analysis of a carcinogen mixture experiment. III. Carcinogens with different target systems, aflatoxin B1, N-butyl-N-(4-hydroxybutyl)nitrosamine, lead acetate, and thiouracil. *Toxicol. Ind. Health* 5:1-23.
- Feigelson, H.S., R.K. Ross, M.C. Yu, G.A. Coetzee, J.K. Reichardt, and B.E. Henderson. 1996. Genetic susceptibility to cancer from exogenous and endogenous exposures. *J. Cell. Biochem. (Suppl.)* 25:15-22.
- Feng, L., R.S. Thomas, D. Ewert, S.A. Saghir, S.A. Benjamin, and R.S.H. Yang. 1998. PBPK/PD model for the toxicologic interactions of three chemicals: Kepone, carbon tetrachloride, and 1,1,2,2-tetrachloroethane. [Abstract]. *The Toxicologist* 42:342.
- Feron, V.J., J.P. Groten, J.A. van Zorge, F.R. Cassee, D. Jonker, and P.J. van Bladeren. 1995. Toxicity studies in rats of simple mixtures of chemicals with the same or different target organs. *Toxicol. Lett.* 82/83:505-512.
- Finney, D.J. 1964. *Statistical Methods in Biological Assays*, 2nd Ed. London: Charles Griffin.
- Friedman, A., D. Kaufer, J. Shemer, I. Hendler, H. Soreq, and I. Tur-Kaspa. 1996. Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. *Nature Med.* 2:1382-1385.
- GAO (United States General Accounting Office). 1998. Chemical Weapons. DOD Does Not Have a Strategy to Address Low-Level Exposures. United States General Accounting Office Report to Congressional Requesters, GAO/NSIAD-98-228. Washington D.C.: United States General Accounting Office. (September 1998). 39 pp.
- Gaylor, D. 1998. Safety assessment with hormetic effects. *BELLE Newsletter.* 6:6-8.

- Gennings, C. and W.H. Carter, Jr., 1996. Utilizing concentration-response data from individual components to detect statistically significant departures from additivity in chemical mixtures. *Biometrics* 51:1264-1277.
- Germolec, D.R., R.S.H. Yang, M.P. Ackermann, J.G. Rosenthal, G.A. Boorman, M. Thompson, P. Blair, and M.I. Luster. 1989. Toxicology studies of a chemical mixture of 25 groundwater contaminants: (II) Immunosuppression in B6C3F1 mice. *Fundam. Appl. Toxicol.* 13:377-387.
- Ghoshroy, S., K. Freedman, R. Lartey, and V. Citovsky. 1998. Inhibition of plant viral systemic infection by non-toxic concentrations of cadmium. *Plant J.* 13:591-602.
- Glynn, A.W., Y. Lind, E. Funseth, and N.G. Ilback. 1998. The intestinal absorption of cadmium increases during a common viral infection (coxsackie virus B3) in mice. *Chem. Biol. Interact.* 113:79-89.
- Goldstein, R.S., W.R. Hewitt, and J.B. Hook. 1990. *Toxic Interactions*. San Diego: Academic Press. 488 pp.
- Gouge, S.F., D.J. Daniels, and C.E. Smith. 1994. Exacerbation of asthma after pyridostigmine during Operation Desert Storm. *Mil. Med.* 159:108-111.
- Haley, R.W. 1997. Is Gulf War Syndrome due to stress? The evidence reexamined. *Am. J. Epidemiol.* 146:695-703.
- Haley, R.W., and R. Kurt. 1997. Self-reported exposure to neurotoxic chemical combinations in the Gulf War. A cross-sectional epidemiologic study. *JAMA* 227:231-237.
- Haley, R.W., R. Kurt, and J. Hom. 1997a. Is there a Gulf War Syndrome? Searching for syndromes by factor analysis of symptoms. *JAMA* 227:215-222.
- Haley, R.W., J. Hom, P.S. Roland, W.W. Bryan, P.C. van Ness, F.J. Bonte, M.D. Devous, Sr., D. Mathews, J.L. Fleckenstein, F.H. Wians, Jr., G.I. Wolfe, and T.L. Kurt. 1997b. Evaluation of neurologic function in Gulf War Veterans. A blinded case-control study. *JAMA* 227:223-230.
- Hanin, I. 1996. The Gulf War, stress and a leaky blood-brain barrier. *Nature Med.* 2:1307-1308.
- Hirvonen, A. 1995. Genetic factors in individual responses to environmental exposures. *J. Occup. Environ. Med.* 37:37-43.
- Hong, J.Y. and C.S. Yang. 1997. Genetic polymorphism of cytochrome P450 as a biomarker of susceptibility to environmental toxicity. *Environ. Health Perspect.* 105(Suppl. 4):759-762.
- Hong, H.L., R.S.H. Yang, and G.A. Boorman. 1991. Residual damage to hematopoietic system in mice exposed to a mixture of groundwater contaminants. *Toxicol. Lett.* 57:101-111.
- Hong, H.L., R.S.H. Yang, and G.A. Boorman. 1992. Alterations in hematopoietic responses in mice caused by drinking a mixture of 25 groundwater contaminants. *J. Environ. Pathol. Toxicol. Oncol.* 11:1-10.
- Hong, H.L., R.S.H. Yang, and G.A. Boorman. 1993. Enhancement of myelotoxicity induced by repeated irradiation in mice exposed to a mixture of groundwater contaminants. *Arch. Toxicol.* 7:358-364.
- Hubert, M. and D. Lison. 1995. Study of muscular effects of short-term pyridostigmine treatment in resting and exercising rats. *Human Exp. Toxicol.* 14:49-54.
- Ikawa, S., F. Uematsu, K. Watanabe, T. Kimpara, M. Osada, A. Hossain, I. Sagami, H. Kikuchi, and M. Watanabe. 1995. Assessment of cancer susceptibility in humans by use of genetic polymorphisms in carcinogen metabolism. *Pharmacogenetics* 5 (Spec No):S154-160.
- Inaba, T., D.W. Nebert, B. Burchell, P.B. Watkins, J.A. Goldstein, L. Bertilsson, and G.T. Tucker. 1995. Pharmacogenetics in clinical pharmacology and toxicology. *Can. J. Physiol. Pharmacol.* 73:331-338.
- IOM (Institute of Medicine). 1995. *Health Consequences of Service During the Persian Gulf War: Initial Findings and Recommendations for Immediate Action*. Washington, D.C.: National Academy Press.
- IOM (Institute of Medicine). 1996. *Interactions of Drugs, Biologics, and Chemicals in U. S. Military Forces*. Washington, D.C.: National Academy Press.
- Jamal, G.A. 1997. Neurological syndromes of organophosphorus compounds. *Adverse Drug React. Toxicol. Rev.* 16:133-170.
- Jamal, G.A. 1998. Gulf War Syndrome — a model for the complexity of biological and environmental interaction with human health. *Adverse Drug React. Toxicol. Rev.* 17:1-17.
- Jamal, G.A., S. Hansen, F. Apartopoulos and A. Peden. 1996. The 'Gulf War Syndrome.' Is there evidence of dysfunction in the nervous system? *J. Neurol. Neurosurg. Psychiat.* 60:449-451.
- JCS (Joint Chiefs of Staff). 1998. Memorandum : Deployment Health Surveillance and Readiness. MCM-251-98. Office of the Chairman, Washington, D.C. Dated 04 December 1998.
- Johnson, T.E., and H. Bruunsgaard. 1998. Implications of hormesis for biomedical aging research. *BELLE Newsletter.* 6(3):17-19.
- Jonker, D., R.A. Woutersen, and V.J. Feron. 1996. Toxicity of mixtures of nephrotoxicants with similar or dissimilar mode of action. *Food Chem. Toxicol.* 34:1075-1082.
- Kelly, C. and J. Rice. 1990. Monotone smoothing with application to dose-response curves and the assessment of synergism. *Biometrics* 46:1071-1085.

- Kligerman, A.D., R.E. Chapin, G.L. Erexson, D.R. Germolec, P. Kwanyuen, and R.S.H. Yang. 1993. Analyses of cytogenetic damage in rodents following exposure to simulated groundwater contaminated with pesticides and a fertilizer. *Mutat. Res.* 300:125-134.
- Kourakis, A., M. Mouratidou, A. Barbouti, and M. Dimikiotou. 1996. Cytogenetic effects of occupational exposure in the peripheral blood lymphocytes of pesticide sprayers. *Carcinogenesis* 17:99-101.
- Kuo, J.C.W., and J. Wei. 1969. A lumping analysis in monomolecular reaction systems: analysis of approximately lumpable system. *Ind. Eng. Chem. Fundam.* 8:124-133.
- Landrigan, P.J. 1997. Illness in Gulf War Veterans. Causes and consequences. *JAMA* 227:259-261.
- Lazarou, J., B.H. Pomeranz, and P.N. Corey. 1998. Incidence of adverse drug reactions in hospitalized patients. A meta-analysis of prospective studies. *JAMA* 279:1200-1205.
- Liang, K.Y., and S.L. Zeger. 1986. Longitudinal data analysis for discrete and continuous outcomes. *Biometrics* 42:121-130.
- Lindenschmidt, R.C., and H.P. Witschi. 1990. Toxicological interactions and other target organ toxicities. Pp. 409-442. In: *Toxic Interactions*, R.S. Goldstein, W.R. Hewitt, and J.B. Hook, eds. San Diego: Academic Press.
- Llback, N.G., J. Fohlman, and G. Friman. 1993. Altered distribution of heavy metals and lipids in coxsackievirus B3 infected mice. *Scand. J. Infect. Dis. (Suppl.)* 88:93-98.
- Llback, N.G., J. Fohlman, and G. Friman. 1994a. Changed distribution and immune effects of nickel augmented viral-induced inflammatory heart lesions in mice. *Toxicology* 91:203-219.
- Llback, N.G., J. Fohlman, G. Friman, and A. Ehrnst. 1994b. Immune responses and resistance to viral-induced myocarditis in mice exposed to cadmium. *Chemosphere* 29:1145-1154.
- Llback, N.G., J. Fohlman, G. Friman, and A.W. Glynn. 1992. Altered distribution of 109 cadmium in mice during viral infection. *Toxicology* 71:193-202.
- Llback, N.G., U. Lindh, J. Fohlman, and G. Friman. 1995. New aspects of murine coxsackie B3 myocarditis — focus on heavy metals. *Eur. Heart J.* 16 (Suppl 0):20-24.
- Lodish, H., D. Baltimore, A. Berk, S.L. Zipursky, P. Matsudaira, and J. Darnell. 1995. Pp. 885. In: *Molecular Cell Biology*. Third Ed. New York: W. H. Freeman & Company.
- Loewenstein-Lichtenstein, Y., M. Schwarz, D. Glick, B. Nørgaard-Pedersen, H. Zakut, and H. Soreq. 1995. Genetic predisposition to adverse consequences of anti-cholinesterases in 'atypical' BCHE carriers. *Nature Med.* 1:1082-1085.
- Machado, S.G., and G.A. Robinson. 1994. A direct, general approach based on isobolograms for assessing the joint action of drugs in pre-clinical experiments. *Stat. Med.* 13:2289-2309.
- McCabe, M., and M. Nowak. 1986. Synergistic modulation of lymphocyte mitogenesis by carcinogenic xenobiotics. *Bull. Environ. Contam. Toxicol.* 37:187-191.
- McConnell, R., A.T. Fidler, and D. Chrislip. 1986. Health Hazard Evaluation Determination. Report No. 83-085, NIOSH, U. S. Department of Health and Human Services. Washington, D.C.
- McFadden, S.A. 1996. Phenotypic variation in xenobiotic mechanism and adverse environmental response: focus on sulfur-dependent detoxification pathways. *Toxicology* 111:43-65.
- Meyer, U.A., and U.M. Zanger. 1997. Molecular mechanisms of genetic polymorphisms of drug metabolism. *Annu. Rev. Pharmacol. Toxicol.* 37:269-296.
- Morré, D.J. 1998. A protein disulfide-thiol interchange protein with NADH: Protein disulfide reductase (NADH oxidase) activity as a molecular target for low levels of exposure to organic solvents in plant growth. *BELLE Newsletter* 6(3):25-33.
- Morse, J.G. 1998. Agricultural implications of pesticide-induced hormesis of insects and mites. *BELLE Newsletter.* 6:20-23.
- Novick, S.G., J.C. Godfrey, R.L. Pollack, and H.R. Wilder. 1997. Zinc-induced suppression of inflammation in the respiratory tract, caused by infection with human rhinovirus and other irritants. *Med. Hypotheses* 49:347-357.
- NRC (National Research Council). 1994a. Pp. 58, 104, 92-105. In: *Science and Judgment in Risk Assessment*. Washington, D.C.: National Academy Press.
- NRC (National Research Council). 1994b. *Health Effects of Permethrin-Impregnated Army Battle-Dress Uniforms*. Washington, D.C.: National Academy Press. 227 pp.
- PDR. 1996. *PDR Guide to Drug Interactions and Side Effects Indications*. Montvale, NJ: Medical Economics Co. 1553 pp.
- Pelekis, M., and K. Krishnan. 1997. Assessing the relevance of rodent data on chemical interactions for health risk assessment purposes: A case study with dichloromethane-toluene mixture. *Regul. Toxicol. Pharmacol.* 25:79-86.
- Pollak, J.K. 1993. *The Toxicity of Chemical Mixtures*. The Centre for Human Aspects of Science and Technology. Sidney, Australia: The University of Sidney. 77 pp.

- Porter, W.P., R.D. Hinsdill, A. Fairbrother, L.J. Olson, J. Jaeger, T. Yuill, S. Bisgaard, W.G. Hunter, and K. Nolan. 1984. Toxicant-disease-environment interactions associated with suppression of immune system, growth, and reproduction. *Science* 224:1014-1017.
- Portier, C.J., D.G. Hoel, N.L. Kaplan, and A. Kopp. 1990. Biologically based models for risk assessment. Pp. 20-26. In: *Complex Mixtures and Cancer Risk*, H. Vainio, M. Sorsa, and A.J. McMichael, eds. Lyon, France: IARC.
- Pott, W.A., S.A. Benjamin, and R.S.H. Yang. 1998. Antagonistic interactions of arsenic-containing mixtures in a multiple organ carcinogenicity bioassay. *Cancer Lett.* 133:185-190.
- Pott, W. A., S.A. Benjamin, and R.S.H. Yang. 1999. Antagonism of hepatic preneoplastic foci by an arsenic-containing mixture: Role of apoptosis. [Abstract]. *The Toxicologist* 48:346.
- Prentice, R.L., R.T. Smythe, D. Krewski, and M. Mason. 1992. On the use of historical control data to estimate dose response trends in quantal bioassay. *Biometrics* 48:459-478.
- Purcell, K.J., G.H. Cason, M.L. Gargas, M.E. Andersen, and C.C. Travis. 1990. In vivo metabolic interactions of benzene and toluene. *Toxicol. Lett.* 52:141-152.
- Quann, R.J. 1998. Modeling the chemistry of complex petroleum mixtures. *Environ. Health Perspect.* 106:1441-1450.
- Quann, R.J., and S.B. Jaff. 1996. Building useful models of complex reaction systems in petroleum refining. Plenary Paper. *Chem. Eng. Sci.* 51: 1615-1635.
- Rao, V.R., Y.T. Woo, D.Y. Lai, and J.C. Arcos. 1989. Database on promoters of chemical carcinogenesis. *Environ. Carcinogenesis Rev.* C7:145-386.
- Rannug, A., A.K. Alexandrie, I. Persson, and M. Ingelman-Sundberg. 1995. Genetic polymorphism of cytochromes P450 1A1, 2D6 and 2E1: regulation and toxicological significance. *J. Occup. Environ. Med.* 37:25-36.
- Raunio, H., K. Husgafvel-Pursiainen, S. Anttila, E. Hietanen, A. Hirvonen, and O. Pelkonen. 1995. Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility — a review. *Gene* 159:113-121.
- Rhim, J.S., G. Jay, P. Arnstein, F.M. Price, K.K. Sanford, and S.A. Aaronson. 1985. Neoplastic transformation of human epidermal keratinocytes by Ad12/SV40 and Kirsten sarcoma viruses. *Science* 227:1250-1252.
- Rook, G.A.W., and A. Zumla. 1997. Gulf War Syndrome: is it due to a systemic shift in cytokine balance towards a Th2 profile? *Lancet* 349:1831-1833.
- Safe, S.H. 1998. Hazard and risk assessment of chemical mixtures using the toxic equivalency factor approach. *Environ. Health Perspect.* 106 (Suppl. 4):1051-1058.
- Sato, A., K. Endoh, T. Kaneko and G. Johansson. 1990. Effects of consumption of ethanol on the biological monitoring of exposure to organic solvent vapors: A simulation study with trichloroethylene. *Brit. J. Ind. Med.* 48:548-556.
- Sielken, R.L., and D.E. Stevenson. 1998. Some implications for quantitative risk assessment if hormesis exists. *BELLE Newsletter* 6(3):13-17.
- Srinivas, U., J.H. Braconier, B. Jeppsson, M. Abdulla, B. Akesson, and P.A. Ockerman. 1988. Trace element alterations in infectious diseases. *Scand. J. Clin. Lab. Invest.* 48:495-500.
- Stebbing, A.R.D. 1982. Hormesis—the stimulation of growth by low levels of inhibitors. *Sci. Total Environ.* 22:213-234.
- Stebbing, A.R.D. 1997. A theory for growth hormesis. *BELLE Newsletter* 6:1-11.
- Takayama S, H. Hasegawa, and H. Ohgaki. 1989. Combination effects of fort carcinogens administered at low doses to male rats. *Jpn. J. Cancer Res.* 80(8):732-6.
- Tardif, R., S. Lapare, G. Charest-Tardif, J. Brodeur, and K. Krishnan. 1993. Physiologically-based modeling of the toxicokinetic interaction between toluene and m-xylene in the rat. *Toxicol. Appl. Pharmacol.* 120:266-273.
- Tardif, R., S. Lapare, G. Charest-Tardif, J. Brodeur, and K. Krishnan. 1995. Physiologically-based modeling of a mixture of toluene and xylene. *Risk Anal.* 15:335-342.
- Tardif, R., G. Charest-Tardif, J. Brodeur, and K. Krishnan. 1997. Physiologically-based pharmacokinetic modeling of a ternary mixture of alkyl benzenes in rats and humans. *Toxicol. Appl. Pharmacol.* 144:120-134.
- Teeguarden, J.G., Y. Dragan, and H.C. Pitot. 1998. Implications of hormesis on the bioassay and hazard assessment of chemical carcinogens. *BELLE Newsletter* 6:8-13.
- Thakore, K.N., M.L. Gargas, M.E. Andersen, and H.M. Mehendale. 1991. PBPK derived metabolic constants, hepatotoxicity, and lethality of bromodichloromethane in rats pretreated with chlordecone, phenobarbital or Mirex. *Toxicol. Appl. Pharmacol.* 109:514-528.
- Verhaar, H.J.M., J.S. Morroni, K.F. Reardon, S.M. Hays, D.P. Gaver, R.L. Carpenter, and R.S.H. Yang. 1997. A proposed approach to study the toxicology of complex mixtures of petroleum products: The integrated use of QSAR, lumping analysis, and PBPK/PD modeling. *Environ. Health Perspect.* 105 (Suppl. 1): 179-195.
- Weber, W.W. 1995. Influence of heredity on human sensitivity to environmental chemicals. *Environ. Mol. Mutagen.* 25(Suppl. 26):102-114.

- Wei, J., and J.C.W. Kuo. 1969. A lumping analysis in monomolecular reaction systems: analysis of the exactly lumpable system. *Ind. Eng. Chem. Fundam.* 8:114-123.
- West, W.L., E.M. Knight, S. Pradhan, and T.S. Hinds. 1997. Interpatient variability: genetic predisposition and other genetic factors. *J. Clin. Pharmacol.* 37:635-648.
- Yang, R.S.H. 1994. *Toxicology of Chemical Mixtures: Case Studies, Mechanisms, and Novel Approaches.* San Diego, CA: Academic Press. 720 pp.
- Yang, R.S.H. 1996. Some current approaches for studying combination toxicology in chemical mixtures. *Food Chem. Toxicol.* 34:1037-1044.
- Yang, R.S.H. 1997. Toxicologic interactions of chemical mixtures. Pp.189-203. In: *Comprehensive Toxicology*. Vol. 1, General Principles, Toxicokinetics, and Mechanisms of Toxicity, J. Bond, ed. Oxford, England: Elsevier Science Ltd.
- Yang, R.S.H., R.S. Thomas, D.L. Gustafson, J.A. Campain, S.A. Benjamin, H.J.M. Verhaar, and M.M. Mumtaz. 1998. Approaches to developing alternative and predictive toxicology based on PBPK/PD and QSAR modeling. *Environ. Health Perspect.* 106 (Suppl. 6):1385-1393.
- Yang, R.S.H., L. Feng, and S.A. Benjamin. 1999. Further refinement of a physiologically based pharmacokinetic/pharmacodynamic model for the toxicologic interaction between Kepone and carbon tetrachloride. [Abstract]. *The Toxicologist* 48:281.
- Zbinden, G. 1976. *Progress in Toxicology: Special Topics, Vol. 2.* Berlin: Springer.