

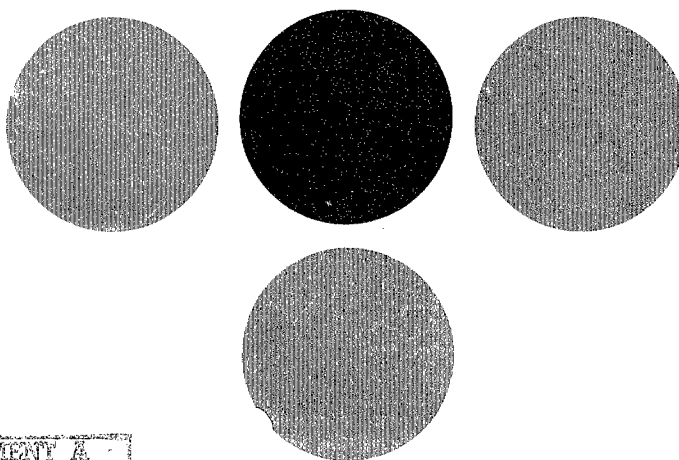
VOL. 111 NOS. 1-3

JULY 17th 1996

ISSN 0300-483X
TXCYAC 111 (1-3) 1-350 (1996)

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An international journal concerned with
the effects of chemicals on living systems
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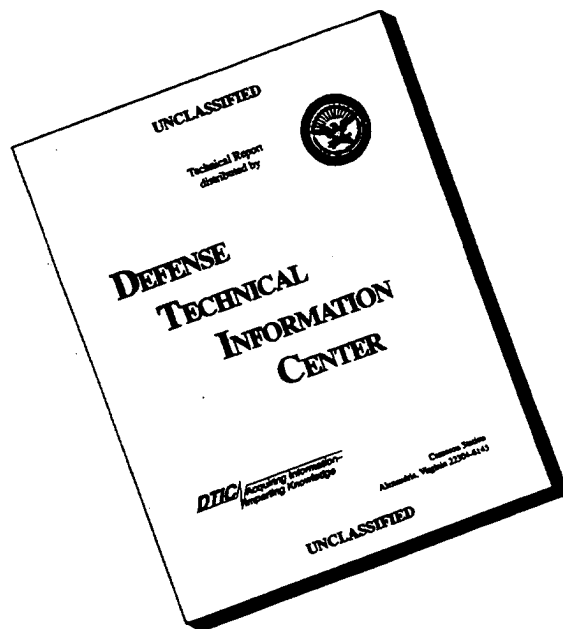
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Special Issue: Risk Assessment Issues for Sensitive Human Populations
Conference Proceedings, Wright Patterson AFB, OH
(April 25-27, 1995)

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TOXICOLOGY

An international journal concerned with the effects
of chemicals on living systems and immunotoxicology

Aims and Scope

Toxicology is a journal for the publication of original scientific papers on the biological effects arising from the administration of chemical compounds, principally to animals, tissues or cells, but also to man. Such compounds include industrial chemicals and residues, chemical contaminants, consumer products, drugs, metals, pesticides, food additives, cosmetics and additives to animal feeding stuffs. Preference will be given to investigations dealing with the mechanisms of action of toxic agents. Papers describing molecular interactions with cellular and genetic processes will be welcomed.

Quantitative toxicological studies will be published that are of relevance to risk assessment and regulatory management of exposure hazards and safety evaluation. This applies particularly to carcinogenicity, mutagenicity, embryotoxicity and related areas, as well as to alternatives to the use of animals in toxicological experimentation. Epidemiological studies bearing toxicological significance to man fall within the scope of the journal. The Editors would also welcome the submission of concise and pertinent reviews on current issues in toxicology.

IMMUNOTOXICOLOGY, a new section of the journal, will include original papers and peer reviews dealing with any adverse interference of chemicals, including drugs, with the immune system: immunosuppression, hypersensitivity, auto-immunity, immune-mediated side-effects of drugs, toxicity of immunomodulating and immunotherapeutic agents.

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Second class postage paid at Newark, New Jersey. Toxicology (ISSN 0300-483X) is published in 30 issues per year, three issues in January, March, May, July, September and November, and two issues in February, April, June, August, October and December by Elsevier Science Ireland Ltd., Bay 15, Shannon Industrial Estate, Shannon, Co. Clare, Ireland. The annual subscription in the USA is \$2394. Toxicology is distributed by Virgin Mailing and Distribution, 10 Camptown Road, Irvington, New Jersey 07111-1105. **Postmaster:** Please send address corrections to Toxicology, c/o Elsevier Science Inc., 660 White Plains Road, Tarrytown, NY 10591-5153.

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0300-483X/1996/\$15.00

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Special Issue

Risk Assessment Issues for Sensitive Human Populations

Conference Proceedings
Wright-Patterson AFB, OH
(April 25-27, 1995)

Edited by

David Mattie

ManTech Environmental Technol. Inc.
P.O Box 31009
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Cited in: Biological Abstracts; CABS (Current Awareness in Biological Sciences); Cambridge Scientific Abstracts; Chemical Abstracts; Current Contents/Life Sciences Excerpta Medica (EMBASE); Environmental Mutagen Information Center; Environmental Periodicals Bibliography; Index Medicus

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Preface

A conference on Risk Assessment Issues for Sensitive Human Populations was held in Dayton, OH, 25-27 April 1995. The sponsors were Tri-Service Toxicology, Wright-Patterson Air Force Base; the Office of Research and Development, U.S. Environmental Protection Agency; and the Division of Toxicology, ATSDR. Coordination of the conference was provided by ManTech Environmental Technology, Inc., under the terms of Contract No. F33615-90-C-0532 with the Department of the Air Force.

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Introduction

Summary of the Conference on Risk Assessment Issues for Sensitive Human Populations

John M. Frazier*^a, David R. Mattie^a, W. Bruce Peirano^b

^a*Tri-Service Toxicology, Wright-Patterson Air Force Base, Dayton, Ohio, USA*

^b*National Center for Environmental Assessment, U.S. Environmental Protection Agency, Cincinnati, Ohio, USA*

The Conference on Risk Assessment Issues for Sensitive Human Populations was held on 25-27 April 1995, at the Hope Hotel and Conference Center, Wright-Patterson Air Force Base, Dayton, Ohio, USA. The overall theme of this conference, risk assessment, has been the focus of this annual conference series for several years, including the 1993 conference on the Risk Assessment Paradigm After Ten Years and the 1994 conference on Temporal Aspects in Risk Assessment for Noncancer Endpoints. The purpose of the 1995 conference was to present the latest theories, concepts and experimental results in three areas: (1) identification of sensitive populations to be taken into consideration by the risk assessment process, (2) understanding of the biological basis of individual differences and species variability, and (3) techniques to incorporate subpopulation sensitivities into hazard identification, quantitative dose-response relationships and exposure characterization. Support for this conference was provided by Tri-Service Toxicology, Wright-Patterson Air Force Base, Dayton, Ohio, the Office of Research and Development, United States Environmental Protection Agency,

Cincinnati, Ohio, and the Division of Toxicology, Agency for Toxic Substance and Disease Registry, Atlanta, Georgia, with the cooperation of the Committee on Toxicology, National Research Council. This unique combination of supporting agencies created a forum for research scientists, risk assessment practitioners and customers of risk assessments to meet, communicate, and together review the major issues relating to the emerging and difficult problem of providing adequate safety margins for special subpopulations.

The conference was divided into five major sessions: (1) Biological Variability in Risk Assessment for Human Populations, (2) Multiple Chemical Sensitivity: Clinical, Experimental and Theoretical Considerations, (3) Responses of Special Human Subpopulations to Toxicants, (4) Occupational and Environmental Exposure Case Studies, and (5) Incorporating Susceptibility into Risk Assessment. In addition, there was a poster session the first evening and a database/software session the second evening.

The first session introduced the general concepts of chemical sensitivity in human populations. The impact of biological variability in the human population on the risk assessment process was explored. Various factors, such as age, gender, nutrition, genetics, socio-economic condi-

*Corresponding author.

tions, and lifestyle choices, were identified and their influence on biological variability discussed.

The second session was devoted to the issue of multiple chemical sensitivity (MCS). The status of MCS as a symptom, a syndrome or a pathological mechanism is unresolved. The possibility was explored that MCS is a catchall term for a new class of pathologies. From the discussions, it was apparent that we are in the observational stage with respect to understanding MCS. However, a clinical database is being generated. Human studies were described involving double-blind, placebo-controlled chemical challenges in environmentally controlled hospital units and human sensitization studies exploring time-dependent sensitization. Animal models were also presented that focused on induction of neural sensitization and cholinergic supersensitivity. These research efforts are leading the way towards a clearer picture of the pathophysiological and psychological conditions underlying this controversial condition.

Various special population studies were discussed in the third session. Issues ranging from personal choice factors, e.g. smoking and nutrition, to socio-economic constraints, e.g. environmental justice, were considered. Chemical specific studies, including manganese, uranium and methylmercury, illustrated special populations at risk, particularly children. The interactions between chemical toxicity and nutrition were of particular note.

The fourth session provided a forum for the discussion of special populations in the occupational arena. The topic of occupational asthma was highlighted in this session. In addition, the exposure component of the risk assessment process was explored in this session. Biomarkers for exposure are an important component of new strategies for population studies. This session included a discussion of the military exposure issues.

The final session focused on methods and approaches for incorporating differential susceptibility into the risk assessment process. Factors contributing to susceptibility were reviewed in the context of how these factors have been taken into consideration historically. Examples

of chemicals for which information is available on subpopulation susceptibilities were presented. The unique susceptibility of the reproductive system and the developing organism were discussed along with genetic factors that may change or modify susceptibility. This session highlighted the importance of including these factors in the risk assessment process to ensure adequate protection for specific populations with increased sensitivity to chemicals.

The organizers would like to thank all the participants for a successful conference. The conference session co-chairs, Bob Sonawane, Jewell Wilson, Claudia Miller, John Rossi III, Kathryn Mahaffey, Rogene Henderson, David Reisman, Patrick Callaghan, Carole Kimmel, Clay Miller, Colleen Lovett and Nancy Bauer, should be congratulated for bringing together a remarkable collection of experts on this important topic. Without the donation of the time and effort of these individuals the conference would not have been possible. A special thanks to Jim Stokes, ManTech Environmental Technology, Inc., and Sgt. Paul Bloomer, Tri-Service Toxicology, for technical assistance. Their computer expertise was a major factor contributing to the success of the Database/Software session.

The preparation of this volume of peer-reviewed manuscripts required long hours of work by many individuals. We would like to particularly recognize those participants in the conference who contributed manuscripts for inclusion in this volume. In these days of limited resources it is particularly difficult to find time to prepare written documentation of conference presentations and we appreciate the authors' efforts. The editors would like to recognize our colleagues who reviewed these manuscripts and provided many critical comments that have improved these proceedings. We thank them for their efforts. However, to be perfectly honest, this journal volume would not exist without the dedication of Shelia Brooks of ManTech Environmental Technology, Inc. Her management of the production logistics for these proceedings has been invaluable and she deserves our most sincere thanks.

SESSION I
BIOLOGICAL VARIABILITY IN RISK ASSESSMENT
FOR HUMAN POPULATIONS



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TOXICOLOGY

Human interindividual variability in susceptibility to toxic effects: from annoying detail to a central determinant of risk

Dale Hattis

Marsh Institute Center for Technology, Environment and Development (CENTED), Clark University, 950 Main Street, Worcester, MA 01610, USA

Abstract

It is unusual to find variability issues as the central focus of a scientific conference. The discussion below first suggests why variability has often been an "annoying detail" in both basic animal toxicology and the human testing of new drugs. Then it gives some reasons why improved quantitative variability information is likely to be important. Better definition of the sources and magnitude of variability in susceptibility in the human population is a central issue for, (1) making more quantitative estimates of both cancer and non-cancer risks from occupational and environmental exposures, and (2) designing protocols for the use of drugs that maximize benefits for the risks incurred in a diverse patient population. Finally, it offers some suggestions about how better variability information is to be obtained and/or extracted from existing information.

Keywords: Risk assessment; Risk management; Quantitative; Uncertainty

1. Introduction

This paper focuses on three questions:

- *Why has interindividual variability often been an 'annoying detail'?* Part of the answer is in the major way biologists have traditionally defined what is an interesting or worthy problem for scientific study. The focus has primarily been on

qualitatively establishing causal connections (e.g. between specific toxicants and specific effects), and this initial goal is hampered by within-group variability. Further, the regulatory community has not developed procedures to practically utilize quantitative variability information in arriving at social policy decisions. In part for ideological reasons, some public health advocates have tended to avoid the intricacies of quantitative risk assessment entirely and thus forgone the opportunities to study the issue of how many people might actually be harmed to various degrees by exposures above and below typical RfDs by various amounts.

Invited paper presented at the EPA/ATSDR Workshop on Susceptible Subgroups, Wright-Patterson Air Force Base, Dayton, Ohio, April, 1995.

*Corresponding author, Tel: 508 751 4603; Fax: 508 751 4600.

● *Why must we do better?* Practical applications are becoming increasingly important in many subdisciplines of biology. As biological systems are increasingly "engineered" (e.g. desired responses to powerful drugs and hormones), there is an increasing need to answer questions that begin "How much?" "How fast?" and "According to what quantitative functional relationship?" in addition to the basic qualitative causal pathway questions (e.g. does this chemical cause this effect). Moreover, for the practical specialty of human health risk assessment, the newer demands of both the "right" and the "left" in the political spectrum are posing questions that can only be addressed with quantitative variability information. From the "right" come demands for greater accountability in the use of resources for health protection. From the "left" there are demands to more explicitly consider the welfare of "unusual" people.

● *How can we do better?* In part the answer here is a familiar extension of the scientific method. It is useful to break down the complex determinants of variability in susceptibility to toxicants in our diverse population, and study each in turn. Some preliminary results of this kind of decomposition will be presented. In addition there are a number of conceptual and technical reforms in the relevant scientific fields that will be helpful.

2. Why has variability often been an "annoying detail?"

The first priority of experimental toxicology and pharmacology has been to establish basic causal connections: does chemical X cause effect Y? And if so, what is the best experimental or clinical system for reproducibly demonstrating the effect for further study?

In this context, variability in the subjects of experimental or clinical study is an annoyance to be rid of to the maximum extent feasible. The more the variability, the larger the sample size required to demonstrate differences between the effects of experimental and control exposures at an acceptable level of statistical confidence.

Variability is therefore deliberately restricted in most animal experimental work by, (A) genetic homogeneity, (B) use of only a single age class of subjects ("young adults" usually), and often (C) use of a single gender of animals. The groups of animals usually have been subject to relatively uniform environmental stimuli in their previous lives, including (D) uniform and unchanging diet. There also are strong efforts (which should not be weakened) to (E) maintain healthy animal colonies, as free of infections as possible. Of course that exposes the animals to less than the diversity of living conditions that you would expect in wilder, outbred populations. And of course, there are also (F) no deliberate neuroactive drug exposures (e.g. narcotics, alcohol, tobacco, caffeine) unless they are explicit subjects of experimental study. To the extent that such exposures change the baseline state of neurological function in an appreciable portion of people, then people necessarily are exposed to potential environmental neurotoxicants with a different and broader distribution of functional reserve capacities for doing different tasks than the experimental animals used for testing potentially neuroactive agents.

There are similar pressures in the clinical investigation of new pharmaceuticals in people (Hattis, 1994). Here the primary objective is to reject the "null hypothesis" that the drug is completely useless (at some prescribed level of statistical confidence) at dose levels that do not immediately produce unacceptable toxicity. As a consequence, there tends to be somewhat limited investigation of the dose-response functions for both beneficial and adverse effects, and the range of types of patients studied is often limited by stringent exclusion criteria relating to diagnosis, complicating conditions, and sometimes gender. There is thus only a limited opportunity to optimize therapeutic protocols for diverse patient populations during premarket screening. And after drugs are admitted to the market, there are only modest incentives for expensive further research in the form of major clinical studies of the variability in practical "compliance" with prescribed drug use protocols, and the extent of

pharmacokinetic and pharmacodynamic variability in normal clinical use.

A final difficulty is that there has not been much of a "regulatory market" for improved information on human variability in likely susceptibility to different kinds of toxicity from specific agents.

- For non-cancer agents, the primary mode of analysis is still descended from the Lehman and Fitzhugh (1954) "100-fold safety factor" paper. Despite the modern adaptations in terminology (e.g. to "uncertainty factors" rather than "safety factors"), the addition of "modifying factors," the elaboration of RfCs in addition to RfDs, and the use of "benchmark dose" procedures to interpret the animal data, there is still no defined or obvious way to utilize relevant quantitative information on the human variability in either pharmacokinetics or pharmacodynamics to modify RfDs, ADIs or other regulatory levels. Without such procedures to utilize these kinds of relevant information in determining regulatory levels, neither the corporate sponsors of new chemicals nor the regulatory agencies themselves see much incentive to collect and analyze these data.

- Existing risk assessment procedures for carcinogens are intended to be "conservative" in the uncertainty dimension — giving estimates that are expected to be usually higher than true risks for typical people. However these procedures do not consider the likely variability in susceptibility among individual people (National Research Council, 1994). Moreover, although inter-individual variability in exposures is extensively modeled in many risk assessment applications, the risk distributions presented to decision-makers and the public do not generally include any spreading due to likely differences in individual human susceptibility to specific types of carcinogens due to differences in metabolism or other factors. Creating such risk distributions integrating variability in both exposure and susceptibility involves an additional step in analysis and also will require decision makers to face some additional complexity in understanding the consequences of alternative policies.

3. Why must we do better?

The previous section tried to explain why quantitative variability information has been relatively neglected by both basic biological scientists and the regulatory community. This section argues that the natural developmental trajectory of both biological science/technology and regulatory policy will cause this to change.

3.1. New developments in toxicology and epidemiology are likely to include richer interactions between quantitative theory-building and more sophisticated empirical observations

Quantification of variability is one part of the more general development of a quantitative orientation for toxicology and other biological sciences (Hattis, 1993). Some examples of this more general trend include:

- (1) The use of measurements and models of intermediate parameters to "break open the black box" between exposures and disease. A variety of "biological markers" are now being developed as indicators of various processes along the sequence from exposure to toxic chemicals and biological effects of major concern (National Research Council, 1989a,b). Appropriate use of these requires attention to the issue of biological variability in each step in the causal sequence (Schulte and Mazzukelli, 1991; Schulte and Perera, 1993). And in turn the use of biological markers opens up possibilities to quantify variability for individual steps in a causal sequence (Hattis and Silver, 1993).
- (2) The use of dynamic modeling approaches to understand the rates at which different processes happen, and the effects of various types of feedback controls in biological systems. This includes physiologically-based pharmacokinetic modeling (Ramsey and Andersen, 1984) (which is to be preferred to the "classical" pharmacokinetic modeling that is more derived from a curve-fitting tradition). Such models will, among other things, facilitate interpretation of the variability of individual components of a complex system in terms of the variability in overall susceptibility to adverse effects (Bois et al., 1995).

(3) Computer-based techniques (e.g. Monte Carlo simulation and its variants) that simulate the likely implications of multiple sources of variability or uncertainty for the outcomes of a causal process. No one can keep in his or her head the combined effects of several sources of variability. But these tools allow relatively straightforward assessment of the implications of, for example, the combined effects of variations in drug taking behavior among patients, variations in absorption, and variations in individual patient sensitivity, on the numbers of patients that might be expected to develop beneficial and adverse effects from a drug.

3.2. Practical use of biological findings requires quantitative predictions of effects, not just qualitative understanding of causal pathways

Like chemistry before it, biology is increasingly giving rise to practical applications. And realizing the greatest benefit from such applications involves quantitative "engineering"-like questions. With innovations in molecular biology and elsewhere, we are rapidly enhancing our capability to intervene at many levels of organization in biological systems. To intervene intelligently, we need to know not only where the levers are, but how hard to push or pull. Quantification of interindividual variability in response is one part of this larger need for the development of quantitative applied biology (Hattis, 1993).

For example, it is a wonderful achievement of traditional qualitative biology to isolate the gene for a hormone that could replace a biological function that is too low in people with some genetic disease. That gene can be used to produce the hormone in appreciable quantities by transferring it to a convenient bacterium. But in order to turn that hormone, produced in that way, into a viable commercial product that will improve people's lives, several types of quantitative information are needed:

- Dose response information. What are the relationships between the internal concentration of the hormone, and its desirable and undesirable effects? This in turn depends on a number of critical features of the mechanisms of the hor-

none's action. How tightly does it bind to its receptors on specific "target" cells? How many of those receptors must be bound for how long to trigger the relevant changes within those cells? What are the resulting quantitative relationships between the number and size of hormone doses given during the course of a day, and the frequency of beneficial and adverse responses?

- Practical manufacturing technology. The hormone must be made in reasonable quantities in sufficiently pure form to avoid allergic side-effects at reasonable costs. What are the best bacteria in which to manufacture the hormone, in order to give a good yield of material that is easy to purify?

- Procedures for use. Which patients should receive how much of the hormone initially? How should the hormone's use be modified for individual patients based on data that can be collected on both early responses and concentrations of the hormone in blood? Individual patients can be expected to differ, of course, in the efficiency with which they absorb the hormone, the rate at which they eliminate the hormone from the body, and their sensitivity to the hormone's effects. Procedures for use must take these differences into account to produce the greatest benefit with the least risk for as many patients as possible. This is done very imperfectly in the drug development process at present, and after approval, there are diminished incentives for additional costly research (Hattis, 1994). However with pharmaceutical purchases now done more on a collective basis, the competitive environment may increasingly favor use of drugs with more precisely defined risk benefit tradeoffs for particular types of people.

There are fascinating intellectual challenges in addressing each of these types of problems. And analogous issues are posed by other practical applications of biological science in agriculture, forestry and fisheries management, environmental and occupational health, etc. In the future I believe that developing an appropriately structured equation to represent the quantitative features of a key biological process will be just as central to the advancement of biomedical sciences as identification of the relationship be-

tween a particular physical structure and a physiological function.

3.3. *Political demands from the "right"*

The field of quantitative risk assessment in many ways traces its origin to the mid-1970s reaction to the environmental and occupational health protection laws passed in the early 1970s. President Ford as part of his "Whip Inflation Now" (WIN) program issued an executive order (No. 11821) that major regulatory actions would need to be accompanied by an "Inflation Impact Statement." In parallel with 1970 National Environmental Policy Act requirement for Environmental Impact Statements, this called for an assessment of the likely economic costs of proposed regulations, and an evaluation of whether these costs were justifiable in the light of the amounts of expected health and environmental benefits. This was reinforced by a Supreme Court decision in the benzene case (*Industrial Union Department vs. American Petroleum Institute*, 1980) and similar requirements have been imposed by all subsequent presidents.

Now on its face this was a fair question to be posed to the health/environmental science community. What do you expect to achieve for the societal efforts you wish to be expended in various ways? The difficulty was that the health/environmental scientists, both within regulatory agencies and without, had no generally applicable tools to produce even approximate quantitative responses that could be juxtaposed with the "quantitative" estimates of compliance costs and other economic consequences that the engineers and economists could produce.

Through the late 1970s and early 1980s federal agencies developed standardized procedures for making some relatively conservative point estimates of carcinogenic risks based on the multi-stage mechanistic theory of carcinogenesis (Crump et al., 1976, 1977; U.S. Environmental Protection Agency, 1976, 1986; Interagency Regulatory Liaison Group, 1979; Albert et al., 1985). When combined with conservative procedures for estimating exposures, these approaches form a useful screening tool for relatively rapid assessment of whether specific

exposures are likely to be of appreciable concern under a "significant risk/de minimis risk" framework for risk management policy making (Hutt, 1985; Travis et al., 1987; Rosenthal et al., 1990).

However, it has been noticed by economists and other advocates of cost-benefit considerations in regulatory decision-making that this is not exactly what they had in mind for purposes of regulatory impact analysis (Anon, 1990; Office of Management and Budget, 1990). The intentionally "conservative" procedures designed to screen for potentially significant risks have been labeled as "biased" — and the suggestion has been made that massive misallocation of societal resources has resulted.

The underlying controversy is not really over numerical calculation techniques but over the risk management criteria that the calculations are intended to help implement (Hattis and Minkowitz, 1996). Most of the environmental health and safety legislation was passed primarily to address equity/fairness concerns, such as "It is unfair for some people, by emitting pollutants into the air, water, land, workplace, or food, to surreptitiously and without informed consent impose risks on other people." This is not the same as the welfare economics concern for the reduction in overall societal allocative efficiency traceable to "externalities." A decision currently facing the Congress as part of the risk assessment/management legislation proposed in the Republican Contract with America is whether the health and environmental protection mandates in regulatory enabling acts will be superseded across the board by a cost benefit decision rule. In any event, either by voluntary executive action or mandatory legislation, it appears that estimation of expected societal aggregate health benefits will become more important in regulatory decision making over the next few years.

What does this have to do with interindividual variability? It happens that in most cases both variability and uncertainty distributions are likely to be skewed — with a long tail to the right, as is illustrated for uncertainty in cancer potency estimates for genetically acting agents in Fig. 1 (Hattis and Goble, 1991). In general, lognormal distributions of this type are to be expected to be

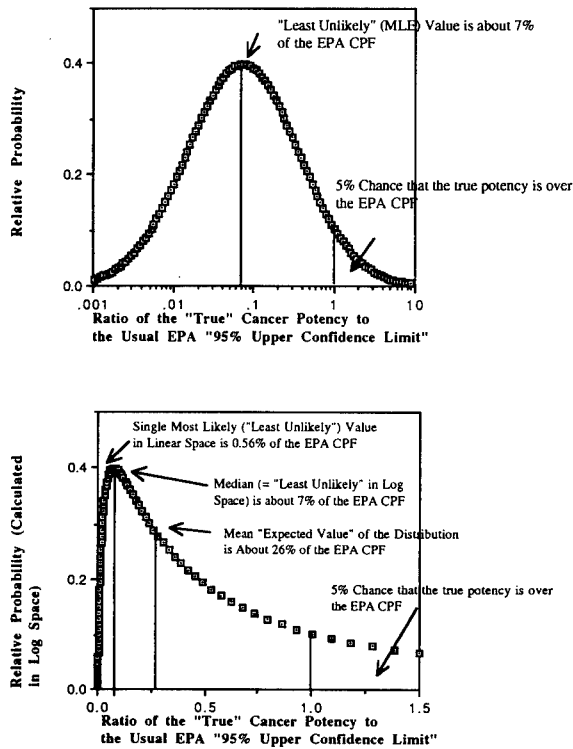


Fig. 1. Estimated likelihood distribution for cancer potencies for genetically-acting carcinogens: (A) log plot, (B) linear plot.

produced when multiple variable or uncertain factors tend to have multiplicative influences on the ultimate parameter of interest (Hattis and Burmaster, 1994). For example, the cancer risks for different individual people may well depend on the product of, (A) the concentration of a substance in a specific environmental medium, (B) individual rates of intake of that medium (e.g. a particular food, drinking water, or air), (C) individual rates of metabolic activation of the substance to DNA reactive forms, and (D) the inverse of the rates of metabolic detoxification, systemic excretion and/or DNA repair.

For a skewed distribution such as that in Fig. 1, the appropriate summary statistic for input into a cost benefit analysis is the arithmetic mean or "expected value" — not either the peak (mode) of the distribution or the 50th percentile (Arrow and Lind, 1970). And in order to calculate the mean, it is vital to know the extent of the skewness (e.g. the geometric standard deviation,

for a lognormal distribution). The counterintuitive result of this is that if our previous tentative estimates of the extent of variability and uncertainty distributions are correct (Hattis et al., 1986; Hattis and Goble, 1991; Hattis and Barlow, 1995), appropriately calculated mean "expected value" estimates of carcinogenic potency for genetically-acting metabolically activated carcinogens may not turn out to be very different in general from the conventionally-assessed EPA 95% upper confidence limit estimates (Hattis and Barlow, 1996). Of course, in addition to this use in quantifying mean risks, any equity-based analysis of the fairness of a risk distribution for an exposed population means facing a three dimensional risk characterization — X level of risk, for the Yth percentile of the population (where Y is the variability dimension), with Z degree of confidence (where Z is the uncertainty dimension) (Hattis and Burmaster, 1994; Hattis and Minkowitz, 1996).

Interindividual variability is of course even more important for making quantitative estimates of non-cancer risks. If quantitative estimation of health benefits becomes necessary in order to promulgate new health protection regulations for traditional toxic effects mediated by individual threshold processes, then the human population distribution of those thresholds becomes a central determinant of the numbers of people likely to be affected by any given exposure (Hattis and Silver, 1994). Unfortunately, procedures have not yet been developed to estimate mean "expected values" for such risks in the face of the major uncertainties in the extent of individual variability for particular classic toxic risks.

3.4. Political demands from the "left"

Where the "right" is seeking to increase the accountability of regulators to the goal of maximizing aggregate economic welfare, there are also voices for increasingly detailed consideration of individual risks from people who think they may be in more jeopardy than usual. The existence of a large amount of interindividual variability for a particular risk can directly raise equity/fairness concerns. This is particularly the case when there is an association between relatively high expo-

tures (and/or risks) and membership in otherwise socially disadvantaged groups (Bullard and Wright, 1993). For example:

- Inner-city African American children have been observed to have much higher blood lead levels, and possible associated risks of developmental impairment, than the general population (Agency for Toxic Substances and Disease Registry, 1993).
- Some ethnic groups (such as members of some Native American tribes) have substantial numbers of people who live by subsistence fishing, and consume much larger amounts of fish than is assumed in standard risk assessment formulae for the general population (Peterson et al., 1994; Columbia River Inter-Tribal Fish Commission, 1994).
- There is concern that unusual dietary patterns and special susceptibility of children has been inadequately reflected in current risk assessment and management procedures (National Research Council, 1993).
- In the drug field, there is increasing recognition that historical exclusion of females and elderly people from early phases of drug testing (Lai et al., 1988) may cause ultimate recommendations for use to be inadequately adapted to the needs of a general population.

Regardless of how the numbers may come out in specific cases, addressing these kinds of specific concerns may be helpful in recognizing that all of these "special" groups have a valued place in our diverse society.

4. How can we do better?

Most of the implications for improvement are implicit in the previous discussion. One important step is better communication and collaboration between experimental biologists and professionals from more mathematically-oriented disciplines. It must become more respectable in biology than it now is to ask "How much?" "How fast?" types of questions, and to use biological data to test, refine and extend quantitative models.

Several reforms are also needed in the current practice of epidemiology. Epidemiologists must become more willing than they now are to depart

from the most parsimonious "black box" models, and include mechanistically relevant intermediate parameters in their study designs. This makes statistical analyses more complicated and more hazardous in some ways, but it has the potential to provide deeper causal insights that are possible in no other way.

And, in a more distressing vein, epidemiologists should join the rest of the scientific community in adopting the convention that once analyses of a particular set of data are published, the underlying data should become available to other investigators for independent analysis. Personally I have been involved in three specific cases (which will not be further detailed here) where access has been denied to the individual data underlying specific published studies which could shed light on interindividual variability or the causation of specific adverse effects. The shared goal of science in seeking truth is fostered by facilitating reproduction of results reported by individual investigators, and the data are in many cases impossible to regenerate independently. Most epidemiological data is a record of individual human tragedies (there are relatively few studies of the determinants of unusually good health outcomes). Access to this private individual information is granted to investigators in the first place with the hope that the investigators will work to prevent similar tragedies for others. And the funds for the investigations generally come from public sources. It is therefore unseemly when those investigators later claim some sort of property right in the resulting data that can be used to prevent others from using it to shed whatever dim light it can on important public health questions.

Beyond this, there is a need to apply traditional scientific techniques to break down human variability into causally relevant components that are amenable to separate study. In earlier work (Hattis and Silver, 1994; Hattis, 1996) individual variability for quantal non-cancer effects has been broken down into three basic categories:

- Uptake. Individual differences in the environmental concentration needed to produce a given intake of toxicant into the body, e.g. due to

Table 1
Observed variability in specific parameters that could be related to susceptibility to carcinogenesis by selected genetically-acting agents^a

Activity subcategory	Number of non-Redundant Datasets	Geometric mean Log(GSD)	Geometric Standard error
<i>Metabolic activation</i>			
P4501A1 and aryl hydrocarbon hydroxylase	5	0.327	1.237
P4502E1 and related activities	3	0.192	1.333
P4503A4 and related activities	5	0.222	1.027
Other oxidizing activities (probable P450)	6	0.308	1.148
Other activities potentially related to metabolic activation	3	0.180	1.080
<i>Detoxification</i>			
Glutathione transferases			
(a) Nonspecific substrate (CDNB)	2	0.161	1.376
(b) GST μ (established polymorphism)	5	0.496	1.091
(c) Other GSTs	4	0.167	1.503
Phase II conjugation reactions	2	0.218	1.099
Superoxide dismutase (anti-oxidant)	1	0.173	
Epoxide hydrolase	1	0.209	
Acetylation (established polymorphism)	1	0.429	
Decrease in mutagenic activity of various direct-acting compounds for <i>Salmonella</i>	3	0.169	1.277
<i>DNA repair</i>			
O ⁶ -Methylguanine methyltransferase	10	0.306	1.085
Uracil DNA glycosylase	3	0.526	1.262
Repair of benzo[<i>a</i>]pyrene DNA adducts	1	0.282	
Unrepaired/misrepaired chromosome damage or cell killing (radiation, etc.)	5	0.172	1.241

^aFrom Hattis and Barlow, 1995, 1996.

differences in breathing rates, dietary habits, etc.

- Pharmacokinetic. Individual differences in the amount of uptake needed to produce a particular concentration-time product of active agent in the blood or at the site of action, e.g. due to differences in metabolic activation or clearance.

- Response or pharmacodynamic. Individual differences in the dose in the blood or at the active site that produces a similar risk of response.

Each of these categories is expressed in an inverted way (e.g. the dose needed to produce a particular response, rather than the response per unit of dose) in order to minimize definitional difficulties arising from nonlinearities in different steps of the causal process.

Finer mechanistic distinctions are also possible. For example, Table 1 shows a breakdown of

observed human variability in metabolic activation, detoxification, and DNA repair activities mediated by specific classes of enzymes (Hattis and Barlow, 1996). This table uses a simple summary measure, the standard deviation of the log₁₀ of the parameter values [the Log(GSD)] to characterize the variability for each data set. The geometric means and standard errors of these values are then given in the last two columns for each type of enzyme. It should be stressed that despite the use of a Log(GSD) to summarize variability here, not all of the underlying distributions are well approximated by a lognormal distribution. This is particularly evident in several cases where an appreciable portion of the variability is determined by known genetic polymorphisms.

Clearly, a fuller understanding of interindividual variability is well within the grasp of current techniques for biological measurement. There must be a concerted effort, however, to develop approaches to analyze and practically use such information to shed light on public policy decisions.

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Toxicology 111 (1996) 15-20

TOXICOLOGY

Kids are different: developmental variability in toxicology

Linda J. Graeter^{*a}, Mary Ellen Mortensen¹

^a*ManTech Environmental Technology, Inc., Tri-Service Toxicology Consortium, 2856 G Street Area B, Bldg. 79, Wright-Patterson Air Force Base, Dayton, OH 45433-7400, USA*

Abstract

Data on the impact of many environmental compounds to human health is often lacking, particularly when considering the risk to the unborn and developing child. The stage of development of the individual at the time of exposure to a toxicant has not always been considered. For example, a higher percentage of ingested lead is absorbed from the gastrointestinal tract of infants than adults. Renal elimination also follows a developmental pattern, being very limited during the newborn period, increasing during infancy and childhood, and declining gradually after puberty. Genetic polymorphisms in metabolic enzyme activity add another dimension of variability. Depending on the particular chemical, this may serve as a protective factor or increase susceptibility to toxic effects (e.g. epoxide hydrolase and fetal hydantoin syndrome). Children also have distinctive behaviors and target organ susceptibilities that warrant special consideration. The consequences of developmental changes are well-known in medical practice, and many drug doses are modified based on age, liver, and renal function, and other factors that may influence pharmacokinetic behavior of drugs. There is a sizable body of such information available, in part, in the pediatric and clinical pharmacology and toxicology literature. The concept of the significance of developmental stage is becoming increasingly important in toxicological risk assessment as well.

Keywords: Children; Unborn; Chemical exposure; Developmental stage; Toxicity; Risk assessment; Variability

1. Introduction

The fetus, infant, and child are becoming an increasingly important focus for exposure and risk assessments in part because they are more sensitive than adults to environmental contaminants, yet many data gaps exist. To be reasonably protective of children's health, risk

assessment needs to consider the biologic factors of the fetus and the biologic, behavioral, and social factors peculiar to infants and children. A schematic of human interaction with various aspects of the environment is shown in Fig. 1. The interactions between the individual and each sphere change with the developmental stage of the person — fetus, infant, toddler, and child. For each of these developmental stages, there are examples of how the spheres impact toxicologic or pharmacologic susceptibility.

*Corresponding author.

¹Nationwide Health Plans, 200 E. Campus View Blvd., Suite 300, Worthington, OH 43235, USA.

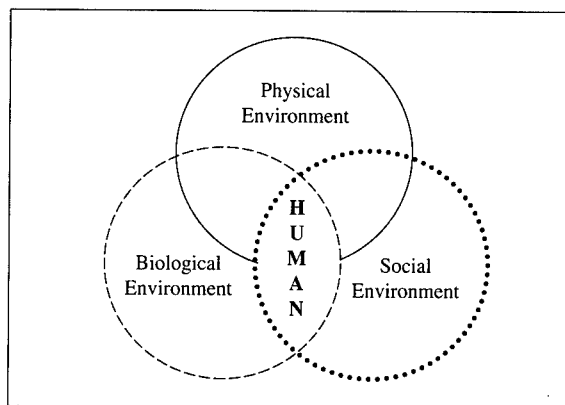


Fig. 1. Schematic representation of the physical, biological, and social environmental factors that affect human development. The interactions of the human being with each sphere change with the developmental stage (age) of the individual.

2. Fetus

In terms of developmental toxicology, normal fetal development depends largely on maternal chemical exposure. The effects of various compounds that cross the placental barrier on the developing fetus are well documented; these effects are of growing concern within the scientific community. *In vivo* animal studies, *in vitro* testing systems, and physiologically based pharmacokinetic models have been used to assess the risk of exposure to a fetus. The fetus incurs additional risk from chemical exposure due to the increased susceptibility of fetal tissues. Fetal tissues are targets for toxicity because the component cells are proliferating, migrating, and differentiating (Rodier, 1994). Rapidly dividing cells provide an opportunity for toxic insults to cause cells to make inaccurate copies of DNA, which can lead to mutations and cancer. In addition, protein is being synthesized rapidly, so errors in this process or in RNA transcription may lead to inadequate or dysfunctional protein substrates or products. The result may be growth impairment or developmental errors. Cells that are migrating can be "misguided" or stopped by toxic insults, with disastrous consequences. Abnormal neuronal migration within the brain has been noted in some cases of psychomotor retardation. Cells

undergoing differentiation can also be "redirected" or halted by a toxic insult. Some teratogens, such as colchicine, appear to operate by this mechanism. The last two processes either do not occur or take place at a considerably reduced pace in adults or fully formed tissues. Consequently, mature tissues may be relatively resistant to some toxic effects, whereas the fetal tissues may be highly susceptible.

Examples of fetal exposure/risk scenarios include environmental tobacco smoke (ETS), lead exposure, and methylmercury poisoning. The effects of environmental tobacco smoke (ETS) on human health are well known. Benzo[*a*]pyrene is one carcinogenic component of ETS (Lofroth, 1989); benzo[*a*]pyrene is metabolized by the P450 cytochrome enzyme system. More than 15 major phase I metabolites have been identified; many of these are further metabolized by phase II enzymes (Smart, 1994). A number of the metabolites are important in the carcinogenic process. First, benzo[*a*]pyrene is metabolized by P450 to an epoxide which is hydrated by epoxide hydrolase to form a dihydriol; this compound is further metabolized to form the ultimate carcinogen, benzo[*a*]pyrene-7,8-diol-9,10-epoxide. This complex is highly mutagenic in eukaryotic cells; it binds readily to DNA forming DNA adducts. The fetus has a relatively low activity of cytochrome P450 (Phase I or oxidative enzymes) and also conjugation or Phase II enzymes (Reed and Besunder, 1989). Consequently, the fetus depends largely on maternal metabolic capabilities. The level of enzymatic activity in this case can either increase or decrease the cancer risk for the fetus and young infant exposed to ETS components. If enzymatic activity is decreased, benzo[*a*]pyrene biotransformation to the ultimate carcinogenic metabolite may not occur and cancer risk may be decreased. If enzymatic activity is increased, complete biotransformation of the parent compound to carcinogenic metabolites might occur, increasing cancer risk.

There are other examples of differences in fetal toxic effects based on variations within the phase I and phase II hepatic and other enzyme systems relating to the concept of genetic polymorphisms—the variability in the biotransformation

of compounds that is due to the genetic makeup of each individual. One such example is fetal hydantoin (phenytoin) syndrome. Teratogenicity has been associated with prenatal administration of the anti-convulsant drug phenytoin; the oxidative metabolites of the compound are ordinarily eliminated by the enzyme epoxide hydrolase (Buehler et al., 1990). Affected fetuses or children lack this enzyme, based on their inherited genetic pattern. This explains why some but not every mother who takes phenytoin for seizures during pregnancy may have an affected newborn (Buehler et al., 1990).

Lead (Pb) easily crosses the placental barrier and accumulates in fetal tissue (Dietrich, 1991). Intrauterine exposure to elevated Pb levels has been associated with decreased gestational maturity, low birth weight, and increased incidence of minor congenital anomalies. Evidence exists that the effect of gestational exposure to Pb extends into the postnatal period to include decreased growth in infancy and early deficits in neurologic and neurobehavioral status (Dietrich, 1991). An animal study using nonhuman primates suggests that the effects of prenatal and early postnatal exposure to low-to-moderate Pb levels persists and become greater over time in the absence of subsequent exposure (Gilbert and Rice, 1987).

Neurotoxicity is a particularly important age-related vulnerability, because the window of susceptibility begins with fetal development and extends through childhood. The brain continues to grow postnatally, with new cells added during the one to two years after birth. Myelination of axons occurs well into the second, and perhaps third year after birth. Cognitive development continues well into adolescence. Methylmercury poisoning is a dramatic well-documented example of neurotoxicity. The dumping of methylmercury into Minamata Bay, Japan, led to the recognition of this environmental teratogen—the second such instance in which environmental contamination produced teratogenic effects (follow-up of atomic bomb survivors led to the recognition of ionizing radiation effects). The disastrous epidemic of methylmercury poisoning in Iraq provided for additional study of fetal and infant exposures

(Bakir et al., 1973). Methylmercury is highly lipid soluble and easily passes through the placenta. Migrating cells in the fetal brain are sensitive to methylmercury, which either arrests or disrupts neuronal migration (Choi et al., 1978). The result of fetal exposure was a newborn who appeared normal, but whose development was not. Blindness, deafness, seizures, and psychomotor retardation were seen in children exposed prenatally to methylmercury (Bakir et al., 1973). Affected brains showed cerebral atrophy and a disrupted pattern of nerve cell bodies, particularly in the cerebellum. Adult brains did not show the same effects. Differential fetal toxicity was also demonstrated when mildly symptomatic or asymptomatic mothers had severely affected babies. In some cases, additional methylmercury exposure occurred through breast milk (Amin-Zaki et al., 1974). In this case, fetal and infant biological susceptibility, combined with environmental and social factors, resulted in devastating, irreversible toxicity.

3. Newborn and early infancy

At this stage of development, the environment again is largely determined by the mother. Breast feeding may continue and can result in exposures to compounds excreted into breast milk, such as lead and PCBs. If the mother or those around her smoke tobacco products, the baby may be exposed to ETS through inhalation. A less familiar exposure route is through dermal absorption. Many agents can be absorbed through the skin; newborns and infants are more susceptible to dermally absorbed compounds than are adults because of a less fully developed barrier function. Two notable incidents that demonstrate increased dermal vulnerability are described below. The first occurred in the 1940s, resulting in several hospital epidemics of methemoglobinemia. Washcloths and diapers used in these nurseries were stamped with an aniline dye. None of the workers who stamped or handled the materials developed toxicity, but enough dye was absorbed through the babies' skin causing the methemoglobinemia which turned them blue (Graubarth et al., 1945). Handwashing and the use of protec-

tive clothing as well as skin characteristics most likely resulted in an insufficient dose and duration of exposure in the adult workers. The infants were also more susceptible to the dye because fetal hemoglobin which is present until about six months of age is unable to reduce oxidized hemoglobin as efficiently as adult hemoglobin (Ross, 1963). In the 1970s, an estimated 7000–10000 babies in Argentina were exposed to phenylmercury, used as a diaper rinse by a commercial laundry (Gotelli et al., 1985). This misuse of the fungicide was discovered when two infants were evaluated for acrodynia, an unusual syndrome that may result from both mercury toxicity and hypersensitivity.

4. Infant and toddler

For older infants and toddlers (approximately six to twenty-four months of age), the environment may be extended beyond the home for longer periods. Oral exploration and locomotion are beginning. Both physiologic and social characteristics can contribute to increased susceptibility at these ages in several ways. Infant lungs have a large absorptive surface area and respire more air than adults because of higher breathing and metabolic rates, thus a larger dose of an airborne toxicant may be absorbed by the infant. Secondly, infant diets are qualitatively and quantitatively different from adults. Cultural traditions and preferences can also influence dietary offerings to young children which may be an important consideration when calculating a safe daily intake of pesticide or other residues on foods.

At this developmental stage, metabolism and excretion may undergo dramatic changes. These processes are reduced or slowed in the fetal and newborn periods, then increase during the first year. By age 8–10, metabolism and excretion reach peak efficiency, and then decline to adult levels around the time of puberty. In pediatrics, the practical impact of these developmental changes is that some drugs have to be dosed less often and in smaller amounts during the newborn period. Premature infants often require smaller and less frequent drug dosages during the new-

born period. Toddlers may need relatively large doses per body weight (kilograms) or more frequent dose intervals than adults (Kacew and Lock, 1989). These same principles most likely extend to chemical exposure.

A child who developed mercury poisoning from latex paint exemplifies some special developmental vulnerabilities of this age (Agocs et al., 1990). The toddler lived in a house heavily painted with latex paint containing excessive amounts of phenylmercuric acetate as a fungicide. Exposure occurred via inhalation of the off-gassed mercury. No other family members were affected, although all had increased urinary mercury excretion as evidence of exposure. Factors that may have increased the child's exposure include the following:

Opportunity: the child spent more time inside the house, especially as he became ill.

Breathing zone: the young child's lower breathing zone may have led to greater mercury exposure, since mercury vapor is heavier than air.

Physiologic factor: the child may have inhaled a greater dose because of higher respiratory and metabolic rates. Younger children also appear to have an increased susceptibility to mercury toxicity. In this case, the child had acrodynia, which is virtually unreported in adults.

5. Child

By the age of two years most young children have developed sufficient independence and curiosity to actively explore their environment. Lead poisoning is an example of how this can be a hazard and also illustrates several factors affecting susceptibility at this age. Hand-to-mouth behavior explains most cases of childhood lead poisoning, either by ingestion of leaded dust which clings to the hands or objects put into the mouth, or by eating leaded dirt or paint chips. At this age, non-food objects often go into the mouth as a way of exploring the environment. Mouthing behavior may also be related to the psychological makeup of a child. For example, children with autism or severe behavior disorders may have extreme and abnormal mouthing, possibly due to a desire for self-stimulation.

The age-related impairments and question of reversibility of lead neurotoxicity in young children are particularly intriguing. Lead effects may depend on the developmental stage or age of the child when exposure occurs. In a small study of children for whom the timing of their lead poisoning could be determined, Shaheen (1984) provided evidence that the timing of exposure determined the nature of the impairments. Specifically, lead poisoning during the second year of life tended to impact the language skills which develop during this time. Children who were lead poisoned at an older age showed impairments in abstract spatial skills, such as designs with blocks.

Evidence suggests several physiologic or biochemical mechanisms for increased susceptibility of young children to lead neurotoxicity. Three of these mechanisms are summarized below:

(1) Brain growth in early childhood includes arborization or branching of neuronal dendrites. This in turn influences the number of synapses available to neurons. In infant animals, lead is known to reduce the number of dendrites per neuron (Bessler and Goldstein, 1991).

(2) Young children are more susceptible to developing acute lead encephalopathy than adults. This may result from physiologic differences in the brain at these ages. Lead appears to alter brain capillary cell function, allowing fluid influx leading to cerebral edema (Goldstein et al., 1974). There may be a higher threshold for this effect in adults.

(3) Lead may interfere with or mimic calcium to activate the intracellular messenger, protein kinase C, an enzyme that seems to participate in brain capillary cell differentiation (Simons, 1993). Activation of this enzyme results in its relocation from cell cytosol to the cell membrane. In immature animals, the enzyme is found in the cytosol, seemingly inactive. In adults, it is bound to the cell membrane in an active form.

These and other studies of the mechanisms of toxicity raise a question of whether lead neurotoxicity is reversible. Clinically, this is an important question because once lead poisoning has occurred chelation therapy may not be effective. Preliminary evidence in children older than one

year suggests that decreased blood lead levels are associated with improved developmental test results in some domains (Ruff et al., 1993).

6. Conclusion

When considering the risk of chemical exposure to the unborn and developing child, toxicologists need to consider the developmental stage at which exposure occurs. This may be a determining factor of both the risk for toxicity and how the exposure may manifest itself later in life. Several examples have been described that illustrate how physical, biological, and behavioral factors contribute to a unique toxicological susceptibility of developing children. Lead poisoning is a well-researched example of how environmental changes (Fig. 1) during development or aging influence the risk and toxicity of exposures throughout a person's life. Children are not small adults. Behavioral and physiologic differences, and how these interact with the physical or social environment may result in age-related toxicologic susceptibility.

Acknowledgements

The authors gratefully acknowledge Dr. Cynthia Bearer. Her presentation "A Developmental Approach to Pediatric Environmental Health" given at the Kids and the Environment Conference, September, 1990, provided inspiration, information and Figure 1 for this review. Debbie Fine provided invaluable editorial and secretarial assistance.

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Toxicology 111 (1996) 21-28

TOXICOLOGY

Quantitative cancer assessment for vinyl chloride: indications of early-life sensitivity¹

Vincent James Cogliano^{*a}, Gerald F.S. Hiatt^b, Arnold Den^b

^aUnited States Environmental Protection Agency, National Center for Environmental Assessment, Washington, DC 20460, USA

^bUnited States Environmental Protection Agency, Region 9, San Francisco, CA 94105, USA

Abstract

Complementary sources of information are analyzed to characterize the early-life cancer risk from inhaling vinyl chloride. A study of partial-lifetime exposures suggests that the lifetime cancer risk depends on age at exposure, with higher lifetime risks attributable to exposures at younger ages. Studies of newborn animal exposures further demonstrate that a brief exposure in newborns can, by the end of life, induce a higher incidence of tumors compared to long-term exposure occurring later in life, including tumor types not induced by exposure later in life. An empirical, quantitative approach is used to model early-life sensitivity to inhaled vinyl chloride, supplementing conventional approaches for estimating the increased cancer risk from lifetime exposure. A single estimate is not presumed to apply to the entire population; instead, the new approach makes distinctions about the cancer risks for different population segments. This assessment shows one way such information might be analyzed, presented, and used to assess actual exposure situations.

Keywords: Vinyl chloride; Early-life sensitivity; Cancer risk; Exposure; Risk assessment

1. Introduction

Cancer risk assessments are generally conducted and applied to a whole population; differential risk assessments for individual subpopulations are usually not available. This stems from

a lack of studies providing information about potentially sensitive subpopulations as well as a lack of methods for assessing differential risks. Vinyl chloride, however, is an example where several innovative laboratory studies provide information about a sensitive period occurring early in life. These studies can be analyzed to more fully characterize the potential for increased early-life risks.

Several sources of information can be brought together to characterize the human cancer risk from inhaling vinyl chloride. Epidemiologic stu-

*Corresponding author.

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dies of workers exposed to vinyl chloride show an increased incidence of angiosarcomas; laboratory studies have induced similar tumors following long-term exposure in three animal species. A study of partial-lifetime exposures in these animal species suggests that the lifetime risk of cancer depends on the age at exposure, with higher lifetime risks attributable to exposures at younger ages. Studies of newborn animal exposures further demonstrate that a brief exposure in newborns can, by the end of life, induce a higher incidence of tumors compared to long-term exposure occurring later in life, including tumor types not induced by exposure later in life.

2. Studies analyzed

ATSDR (1988) cites several reports demonstrating a causal association between occupa-

tional vinyl chloride exposure and various forms of cancer. Most convincing is the association with liver angiosarcoma, a rare form of cancer. Cancer assessments by IARC (1987) and US EPA (1991) found the human evidence sufficient, leading to an overall characterization of vinyl chloride as a known human carcinogen. Studies using laboratory rats, mice, and hamsters confirm the human observations; in particular, liver angiosarcomas were induced in all species. This uncommon demonstration of site concordance makes extrapolation of the animal results to humans especially credible.

In addition to these studies that led to the identification of vinyl chloride as a known human carcinogen, several studies with novel experimental designs provide evidence that early life can be a sensitive period for exposure. Drew et al. (1983) studied the effect of age and duration of vinyl chloride exposure on cancer incidence.

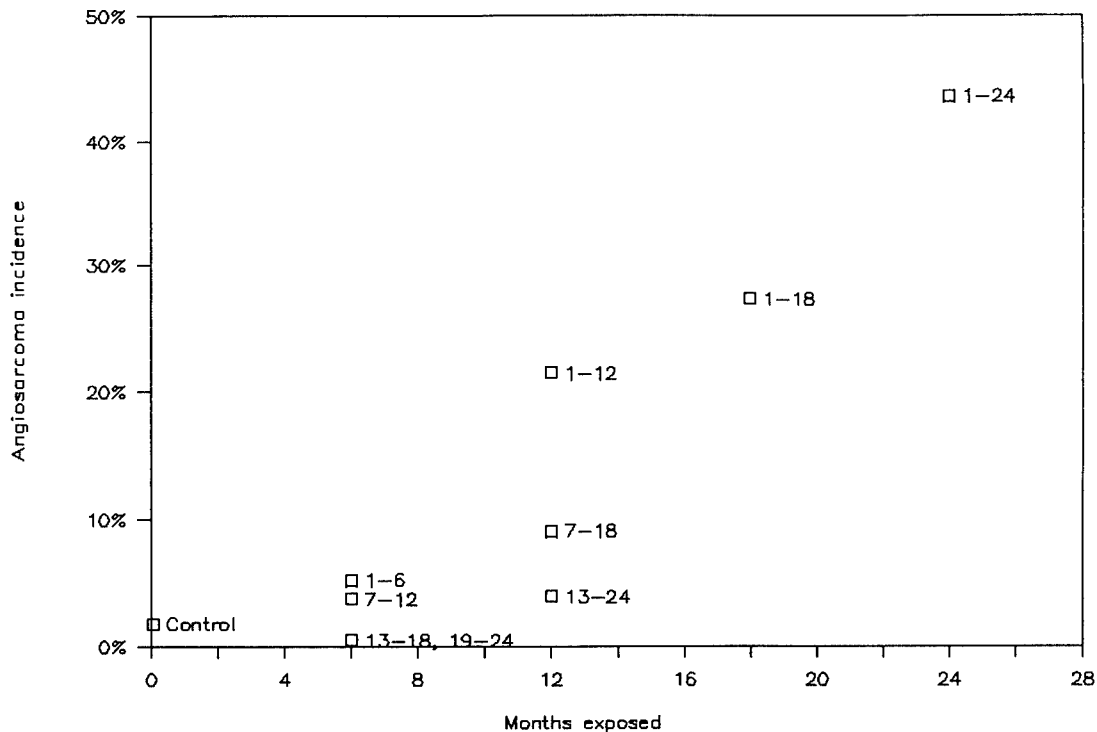


Fig. 1. Effect of duration and age at exposure on angiosarcoma incidence (from Drew et al., 1983).

Groups of female Fischer-344 rats, golden Syrian hamsters, B6C3F1 mice, and CD-1 Swiss mice inhaled vinyl chloride at 100 ppm for durations of 6, 12, 18, or 24 months beginning 0, 6, 12, or 18 months into the exposure period. (Prior to exposure, animals were 5-6 weeks old when received at the testing laboratory, then they were observed and weighed for 3 weeks.) Vinyl chloride caused angiosarcomas and mammary gland carcinomas in all four strains; in addition, there were hepatocellular carcinomas in rats, stomach adenomas and skin carcinomas in hamsters, and lung carcinomas in CD-1 Swiss mice. In general, cancer incidence increased with duration of exposure and decreased the later the age at first exposure. For example, Fig. 1 shows angiosarcoma incidence in each group of rats plotted against exposure duration; incidence increases

with duration, and for the same duration incidence is higher for groups exposed earlier.

Maltoni et al. (1981) investigated the existence of dose-rate effects as part of a comprehensive vinyl chloride study. Groups of male and female Sprague-Dawley rats inhaled 6000 or 10000 ppm vinyl chloride for 100 h under different exposure schedules, three schedules beginning at 13 weeks of age and one schedule beginning at 1 day of age (4 h a day, 5 days a week, for 5 weeks). Fig. 2 shows that angiosarcoma incidence increased sharply in rats exposed from 1 day of age. Fig. 3 compares this angiosarcoma incidence (in the rats exposed for 5 weeks beginning at 1 day of age) to that of rats exposed for 52 weeks beginning at 13 weeks of age; the angiosarcoma incidence for rats exposed for 5 weeks as newborns is higher than that for rats exposed for 52

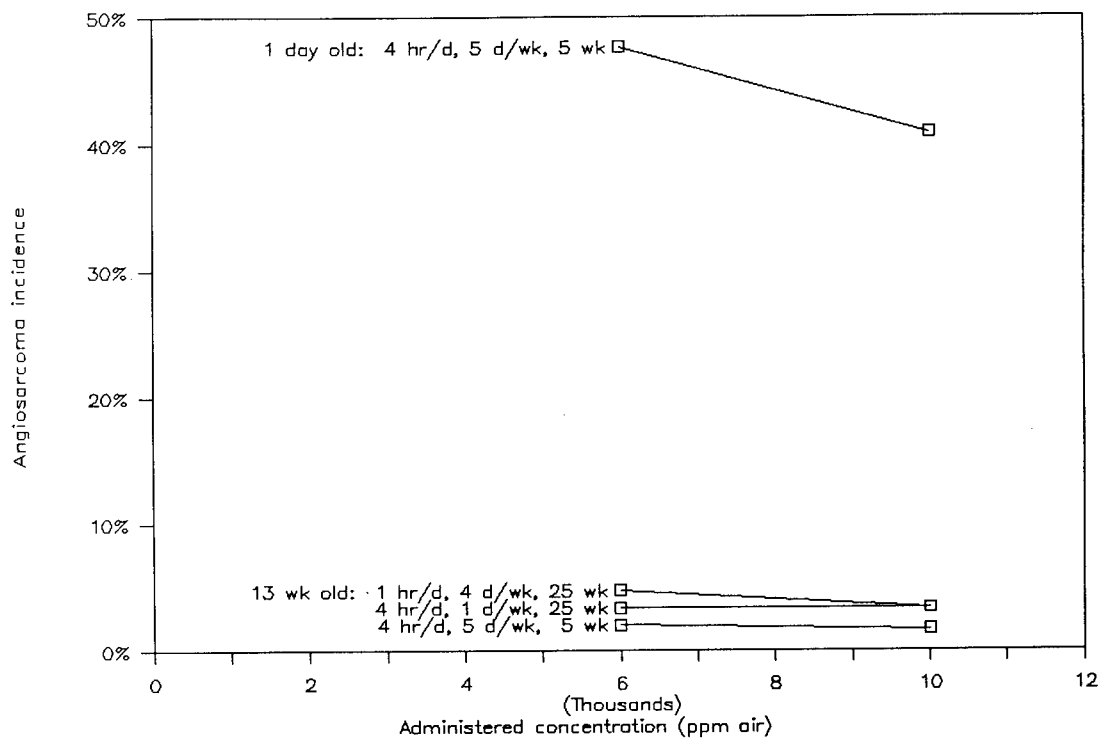


Fig. 2. Effect of age at exposure on angiosarcomas induced by 100-h exposures (from Maltoni et al., 1981, experiments BT10 and BT14).

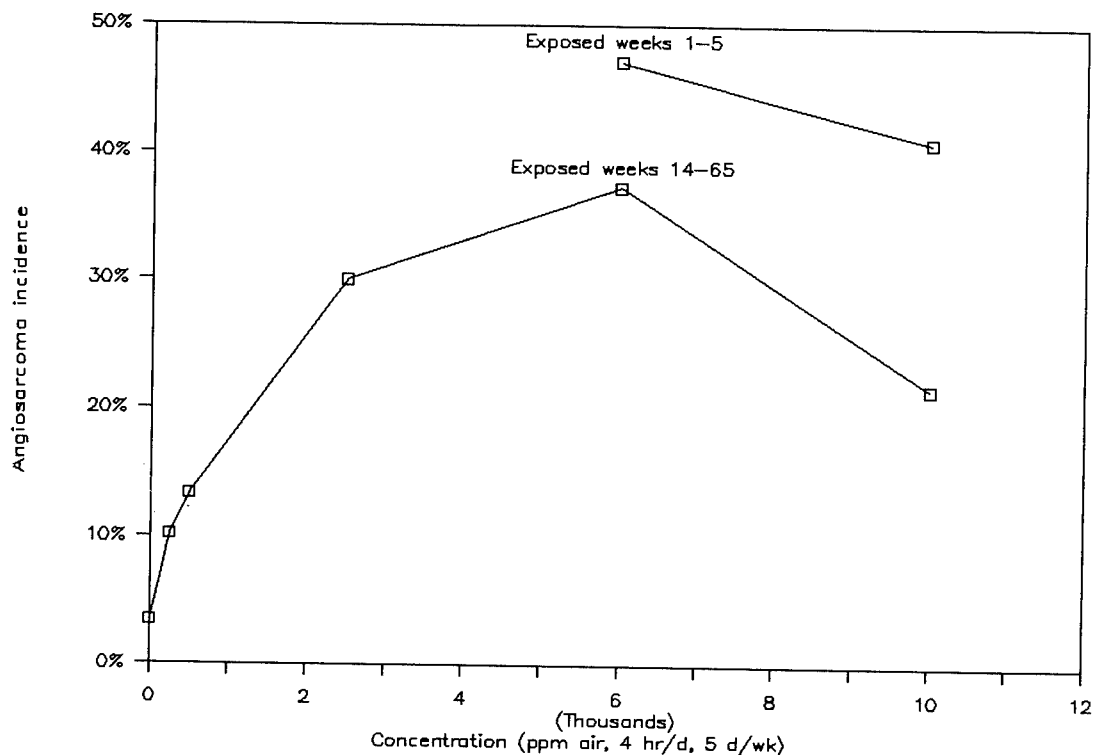


Fig. 3. Comparison of newborn and later-life exposure on angiosarcoma incidence (from Maltoni et al., 1981, experiments BT1 and BT14).

weeks after maturity. Further, hepatoma incidence, virtually nonexistent in rats exposed for 52 weeks after maturity, approaches 50% in rats exposed for 5 weeks as newborns.

Laib et al. (1985) studied the effect of age on induction by vinyl chloride of hepatic adenosine-5'-triphosphatase (ATPase)-deficient enzyme-altered foci, a putative precursor of hepatocellular carcinoma. Groups of newborn male and female Wistar rats inhaled 2000 ppm vinyl chloride for different periods of time; their livers were evaluated at 4 months. The investigators concluded that "the induction of pre-neoplastic hepatocellular lesions in rats by vinyl chloride is restricted to a well-defined period (approximately day 7 to 21) in the early lifetime of the animals." They attributed the lack of response in the first 5 days to the lack of hepatocellular proliferation and the

low rate of vinyl chloride metabolism at this stage of development.

Further mechanistic investigations into the effect of age on vinyl chloride-induced DNA adducts has been studied by Fedtke et al. (1990).

3. Results

An empirical, quantitative approach is used to model early-life sensitivity to inhaled vinyl chloride, supplementing conventional approaches for estimating the increased cancer risk from lifetime exposure. A single estimate is not presumed to apply to the entire population at risk; instead, the new approach makes distinctions about the cancer risks for different population segments.

Fig. 1, where cancer incidence is higher for groups exposed earlier, suggests cancer incidence

might be modeled as a function of remaining lifetime. Cogliano and Parker (1992) analyzed the results of Drew et al. (1983) with an empirical model that approximates cancer risk as a bilinear function of dose and some power of remaining life. That is, let

T be the lifespan, so $T - t$ is the remaining life at age t , and

k be the exponent associated with remaining life.

Then for a dose d received at age t , the incremental cancer risk is assumed proportional to $d(T - t)^k$.

Under this model, the effectiveness of each increment of dose depends on both the dose level d and the age at exposure t . The incremental cancer risk would increase as either the dose d increases or the age at exposure t decreases. To evaluate the effectiveness of a particular expo-

sure schedule, each increment of exposure can be weighted by the proportionality function $d(T - t)^k$ and summed accordingly; for example, if dosing is constant between ages A and B , then the age-weighted exposure for the period of dosing is proportional to

$$d \int_A^B (T - t)^k dt$$

For full life exposure (between ages 0 and T), the age-weighted exposure is proportional to

$$d \int_0^T (T - t)^k dt$$

Expressed as a fraction of full-life exposure, the effectiveness of dosing between ages A and B is,

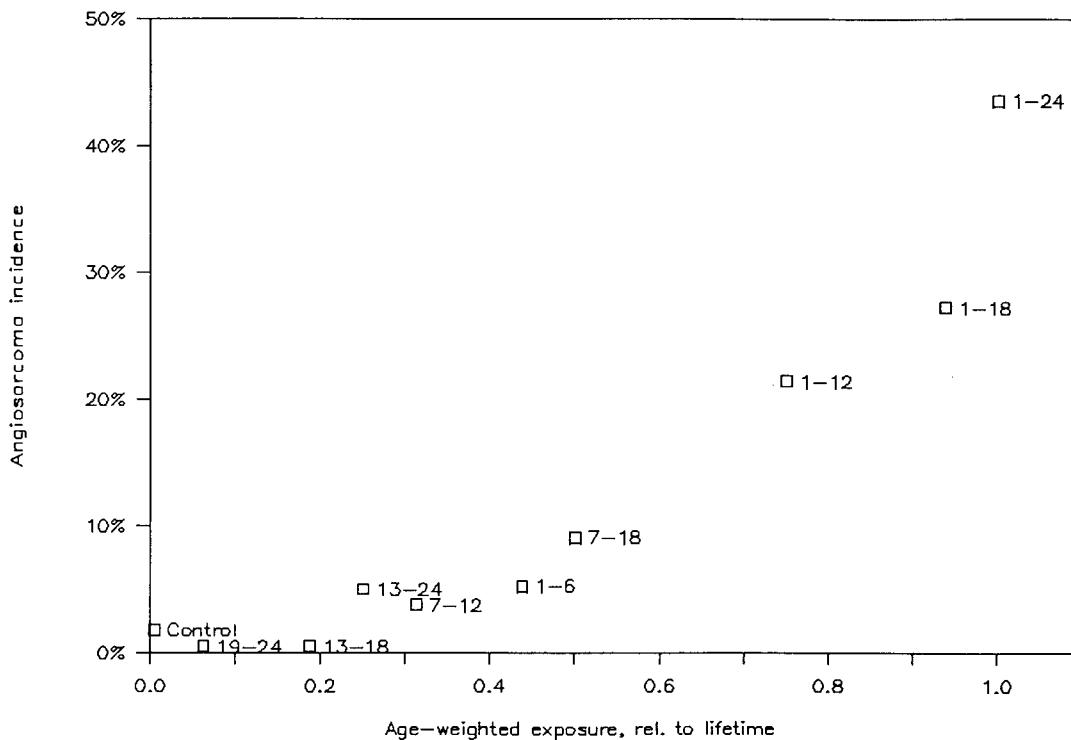


Fig. 4. Data from Fig. 1 replotted to show angiosarcoma incidence as a function of age-weighted exposure ($k = 1$) (from Drew et al., 1983).

therefore,

$$\int_A^B (T-t)^k dt \bigg/ \int_0^T (T-t)^k dt$$

Cogliano and Parker (1992) provide a table showing, for a given duration $B - A$, how age-weighted exposure increases as the age at first exposure A decreases or as k increases above 0. Using age-weighted exposure for $k = 1$ instead of simple duration of exposure, Fig. 4 shows the results from Drew et al. (1983) that were plotted in Fig. 1. In Fig. 4, groups exposed earlier in life are shifted to the right, reflecting a higher age-weighted exposure, while groups exposed later are shifted left. The result resembles a more distinct dose-response curve, suggesting age-weighted exposure is useful in explaining the observed cancer incidences. Thus risk estimates are a function of exposure concentration, exposure duration, and age at exposure. Cancer risks from chronic exposure can be apportioned to different ages, with higher risks for exposure occurring at earlier ages.

These results suggest the potential for vinyl chloride to cause cancer is greatest for newborn exposure. Only the Maltoni et al. (1981) study provides direct information about this sensitive stage of development. Cogliano (1989, 1990) developed risk estimates from the results of Maltoni et al. (1981). The essential features of this analysis

are, (a) the exposure periods in the early-life studies (weeks 1–5 for Maltoni et al. (1981), days 7–21 for Laib et al. (1985)) do not overlap those of the chronic studies (where exposure begins after 2–3 months), (b) the cancer risk from early-life exposure is roughly equal that from full-life exposure beginning after maturity, and (c) because the effects of early-life exposure are different from those of full-life exposure, early-life exposures are treated differently from exposures later in life. Thus, the risk of cancer from vinyl chloride is composed of two parts: a risk from chronic exposure occurring mostly after maturity, and a risk of similar size attributable to short-term, early-life exposure. This effectively doubles the vinyl chloride risk estimate that is derived from chronic studies. The estimate for chronic exposure can be apportioned throughout life according to a declining age-weighting curve described by Cogliano and Parker (1992), while a further risk would be incurred if exposure occurred early in life.

These assessments were considered by the U.S. and California Environmental Protection Agencies to guide their responses to indoor inhaled vinyl chloride in a housing development adjacent to a mixed-use landfill designated as a Superfund site. For cancer risks, a target risk range of 1 in 1 000 000 to 1 in 10 000 was used to determine whether action was taken at the site. Although there is no direct evidence of increased human sensitivity to vinyl chloride-induced carcino-

Table 1
Risk-based action levels for vinyl chloride

Indoor air action level	Response	Risk criterion
< 0.2 ppb	No immediate action	Potential cancer risks from lifetime exposure and from 4-year childhood exposure within target risk range
0.2–1 ppb	Interim (30-day) remediation	Potential lifetime cancer risk from 4-year childhood exposure is at upper end of target risk range
1–25 ppb	Immediate (12-day) remediation	Potential lifetime cancer risk from 4-year childhood exposure exceeds target risk range
25+ ppb	Immediate relocation offered	Potential risk of male reproductive toxicity from subchronic exposure

genicity with exposure during childhood or adolescence, the animal evidence of such an age-dependent sensitivity was considered to be sufficient to warrant public health concern for young children potentially exposed to vinyl chloride. Therefore, cancer risks to young children became the risk focus for development of action levels at low vinyl chloride levels (Hiatt et al., 1994). Noncancer toxicity was also considered; at high exposures a reference dose for male reproductive toxicity could be exceeded (Hiatt et al., 1994). The risk-based action levels are described in Table 1.

4. Discussion

This analysis shows how several innovative study designs can provide information about early-life sensitivity that can be used in risk assessment. The lifetime cancer studies of Drew et al. (1983) and Maltoni et al. (1981), by including some animals with only early-life exposure, provide empirical evidence of enhanced sensitivity. The mechanistic studies of Laib et al. (1985) confirm the empirical evidence of early-life sensitivity. The consistent observation of angiosarcomas in lifetime animal and human occupational studies adds to the credibility of the results. By drawing on these different sources of information, confidence is enhanced in the risk assessment's conclusions.

Besides showing how these innovative study designs can be used in risk assessment, this analysis has implications for the interpretation of epidemiologic studies. Adults may not be the most sensitive population, and hence occupational studies, the predominant study in cancer epidemiology, may not identify hazards to children or other potentially sensitive subpopulations. Quantitative risk estimates based on workers may not be health-conservative for other populations. Management decisions based on worker studies may, thus, not afford the degree of protection anticipated.

There are also implications for study design. Valuable information can be provided by more investigations of the effects of timing of exposure,

effects in immature animals, and epidemiologic studies outside occupational settings. The Maltoni et al. (1981) study design can provide empirical information to identify a potential for early-life sensitivity, and Laib et al. (1985) study shows how mechanistic research can confirm these empirical observations.

Perhaps the most pressing research need, however, is identifying other substances that affect sensitive stages of development. Screening studies need not be prohibitively expensive: the Maltoni et al. (1981) study demonstrates a protocol that can indicate whether newborns are especially susceptible. Valuable information can be gained from a group of animals exposed immediately after birth for a short time. This assessment has shown one way that such information can be analyzed and presented. With this or a similar approach, risk assessments can better identify susceptible individuals most at risk.

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Toxicology 111 (1996) 29-42

TOXICOLOGY

Efficient tissue repair underlies the resiliency of postnatally developing rats to chlordecone + CCl₄ hepatotoxicity

Abraham Dalu, Harihara M. Mehendale*

*Division of Toxicology, College of Pharmacy and Health Sciences, Northeast Louisiana University,
Monroe, LA 71209-0470, USA*

Abstract

It is often assumed that at a younger age populations are at higher risk of toxic effects from exposure to toxic chemicals. Recent studies have demonstrated that neonate and postnatally developing rats are resilient to a wide variety of structurally and mechanistically dissimilar hepatotoxicants such as galactosamine, acetaminophen, allyl alcohol, and CCl₄. Most interestingly, young rats survive exposure to the lethal combination of chlordecone (CD) + CCl₄ known to cause 100% mortality in adult male and female rats. In a study where postnatally developing (20- and 45-day), and adult (60-day) male Sprague Dawley rats were used, administration of CCl₄ (100 µl/kg, i.p.) alone resulted in transient liver injury regardless of age as indicated by plasma alanine transaminase (ALT), sorbitol dehydrogenase (SDH) levels and histopathological lesions. In CD-pretreated rats, CCl₄-induced toxicity progressed with time culminating in 25 and 100% mortality by 72 h after CCl₄ in 45- and 60-day rats, respectively, in contrast to regression of CCl₄-induced toxicity and 0% mortality in 20-day rats. [³H]thymidine (³H-T) incorporation and proliferating cell nuclear antigen (PCNA) studies revealed an association between delayed and diminished DNA synthesis, unrestrained progression of liver injury, and animal death. Time-course studies revealed that the loss of resiliency in the two higher age groups might be due to inability to repair the injured liver rather than due to infliction of higher injury. Intervention of cell division in 45-day CD rats by colchicine (CLC, 1 mg/kg, i.p.) 30 h after CCl₄ challenge increased mortality from 25 to 85%, confirming the importance of stimulated tissue repair in animal survival. In contrast, efficient and substantial DNA synthesis observed in 20-day rats allows them to limit further progression of liver injury, thereby leading to full recovery of this age group with 0% mortality. Examination of growth factors and proto-oncogene expression revealed a 3- and 3.5-fold increase in transforming growth factor-α (TGF-α) and *H-ras* mRNA expressions, respectively, coinciding with maximal hepatocyte DNA synthesis in 20-day normal diet (ND) rats, as opposed to only 2- and 2.5-fold increases observed in 60-day ND rats, respectively. Increased expression of *c-fos* (10-fold) in 20-day rats occurred 1 h after CCl₄ compared to less than a 2-fold increase in 60-day rats. These findings suggest that prompt stimulation of tissue repair permits efficient recovery from injury during early postnatal development of rats.

Keywords: Age-related toxicity; Chlordecone; Carbon tetrachloride; Cell division; Tissue repair; Resiliency

Abbreviations: ALT, alanine transaminase; CD, Chlordecone (Kepone/reg/); CLC, colchicine; ND, normal diet; PCNA, proliferating cell nuclear antigen; SDH, sorbitol dehydrogenase; TGF-α, transforming growth factor-alpha; TGF-β, transforming growth factor-beta.

*Corresponding author, Tel.: (318) 342-1691; Fax: (318) 342-1686; E-mail: PYMEHENDALE/ALPHA.NLU.EDU.

1. Introduction

Age is an important consideration in identifying susceptible population for risk from exposure to chemicals. A greater understanding of the mechanisms underlying the influence of age on the susceptibility to chemical injury is critical in dealing with hepatotoxicity in newborn, young, adults, and elderly. Depending on the chemical, dose, or type of disease, early studies showed that hepatic injury and disease can either increase or decrease with age (Black et al., 1975). Recent studies have demonstrated that neonates and young rats are resilient to a number of hepatotoxicants such as CCl_4 (Dawkins, 1963; Cagen and Klaassen, 1979; Rikans, 1989; Cai and Mehendale, 1993; Dalu et al., 1995a), allyl alcohol (Rikans, 1989), galactosamine (Abdul-Husain and Mehendale, 1992), and acetaminophen (Green et al., 1984). Most interestingly, young rats survive exposure to lethal combination of individually nontoxic doses of chlordecone (CD) + CCl_4 (Cai and Mehendale, 1993; Dalu et al., 1995a), known to cause fulminant hepatic failure (Soni and Mehendale, 1993) and 100% mortality in adult rats (Klingensmith and Mehendale, 1982; Agarwal and Mehendale, 1983; Soni and Mehendale, 1993).

The mechanism of cellular damage caused by CCl_4 has been well established. The compound is bioactivated by cytochrome P-450-mediated reactions to $\cdot\text{CCl}_3$ free radicals (Slater, 1966; 1987; Recknagel and Glende, 1973; Sipes et al., 1974; Mehendale, 1989a,b; 1990), which is further converted to a peroxy radical, $\text{CCl}_3\text{O}\cdot$ (Connor et al., 1986; Mehendale 1989b). There is evidence for covalent binding of CCl_4 upon bioactivation (Slater, 1966, 1987; Sipes et al., 1974; Recknagel and Glende, 1973; Mehendale, 1989a,b; 1990). The free radicals $\cdot\text{CCl}_3$ and $\text{CCl}_3\text{O}\cdot$ readily react with polyunsaturated fatty acids of the endoplasmic reticulum and other hepatocellular membranes to initiate the formation of organic lipid peroxides. In the presence of cellular O_2 , these organic peroxy radicals in turn can react with other polyunsaturated fatty acids to perpetuate a series of self-propagating chain reactions, known as "propagation of lipid peroxidation" (Reck-

nagel and Glende, 1973). Rats receiving a low dose of CCl_4 alone exhibit very limited liver injury as evidenced by histopathological observations at 6 h after the administration of CCl_4 . This injury is progressive up to 12 h, followed by recovery by 24 h (Kodavanti et al., 1989a,b; 1992; Mehendale, 1990). Substantial experimental data suggest that stimulation of hepatocellular regeneration and tissue repair mechanisms play a critical role in the recovery from the limited toxic injury inflicted by a low dose of CCl_4 (Mehendale, 1990; Kodavanti et al., 1992; Chanda and Mehendale, 1995). Dietary exposure to CD (10 ppm in diet for 15 days) is known to potentiate hepatotoxicity and lethality of a low dose of CCl_4 (100 $\mu\text{l}/\text{kg}$) in male (Klingensmith and Mehendale, 1982) and female (Agarwal and Mehendale, 1983) adult rats. Studies have revealed that the CD + CCl_4 combination inhibits CCl_4 -induced liver cell division and tissue repair, thereby permitting progression of injury leading to hepatic failure and death (Curtis and Mehendale, 1980; Klingensmith and Mehendale, 1982; Agarwal and Mehendale, 1983; Lockard et al., 1983a,b; Kodavanti et al., 1989a,b; Mehendale, 1994).

Experimental data suggest that newborns are less sensitive to a number of chemicals like CCl_4 whose toxicity depend upon metabolic activation to a toxic species (Dawkins, 1963; Cagen and Klaassen, 1979). In these studies, age-related differences in cytochrome P-450 levels was offered as a mechanism for lower sensitivity of newborns to CCl_4 toxicity. In contrast to the above studies, Cai and Mehendale (1993) reported that postnatally developing rats were resilient to the lethal combination of CD + CCl_4 . This resiliency is related neither to differences in cytochrome P-450 levels nor to bioactivation-daydependent mechanisms, but is due to enhanced plasticity in hepatocellular regeneration and efficient tissue repair mechanisms in the younger animals (Dalu et al., 1995a).

Recent studies were conducted to investigate the mechanisms underlying the lack of sensitivity to CD + CCl_4 hepatotoxicity and lethality during early postnatal development (Dalu et al., 1995a). These studies indicated that both ongoing (growth-related) and toxicant-stimulated tis-

sue repair were responsible for this marked resiliency. Ongoing cell division is associated with growth of the liver related to animal growth during early postnatal development. Because transforming growth factor- α (TGF- α) (Fausto and Mead, 1989; Michalopoulos, 1990) and proto-oncogenes (Rollins and Stiles, 1988; Fausto and Mead, 1989) are closely related to cell division (and tissue repair), the role of TGF- α and proto-oncogenes in the resiliency of early postnatally developing rats were also investigated. We report here that marked ability of growing livers during early postnatal development to induce compensatory tissue repair enables the rats to restrain toxic injury and restore liver function, leading to recovery from injury. In contrast, quiescence of adult liver hepatocytes leads to unrestrained progression of liver injury, rendering the adults to be highly susceptible to progressive hepatotoxic injury.

2. Age-related differences in chemical-induced hepatic injury and lethality

2.1. Ongoing liver growth during early postnatal development

Growth rates were determined for 1, 5, 10, 20, 35, 45, 60, 80, and 100 days male Sprague-Dawley rats. The changes in liver and body weights in these rats follow the familiar exponential type curves which start to level off around 45 days after birth (Fig. 1). Newborn and young developing rats have actively growing livers (ongoing liver growth), therefore liver growth during postnatal development is linear up to 45 days after birth (Fig. 1). Hence, if liver growth is a factor one would expect to see a phase-out of resiliency around 45-day of age. The ability to replace dead or dying cells would be decreased with age, and as a consequence, chemical-induced liver injury should be expected to progress in an unrestrained manner. These observations indicate that growth rates during postnatal development are steep and linear up to 45 days as evidenced by the increase in liver and total body weight of these rats. The growth rate leveled off after 45 days. These findings impact age-related studies and interpreta-

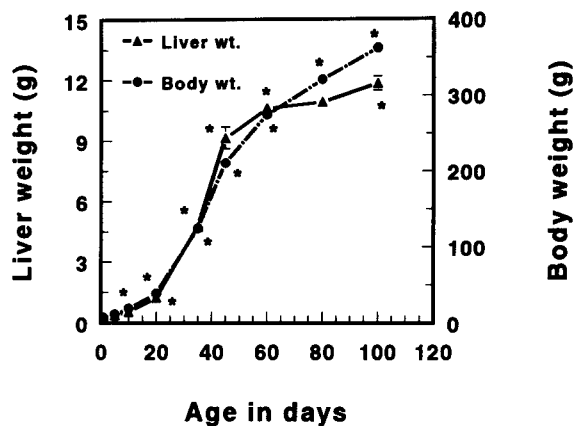


Fig. 1. Growth rate in male Sprague-Dawley rats. Growth rates of liver and total body weight were measured in 1, 5, 10, 20, 35, 45, 60, 80, and 100 day rats. Values are means \pm S.E.M. of 4 rats. *Denotes that liver and body weight were significantly different from the previous age ($P \leq 0.05$).

tions of susceptibilities of younger age populations to toxic chemicals.

2.2. Lethality

The mortality induced by a single nontoxic dose of CCl_4 (100 $\mu\text{l}/\text{kg}$, i.p.) in 20-, 45-, and 60-day-old rats maintained on either ND or 10 ppm CD diet was observed and recorded for 14

Table 1
 CCl_4 -induced lethality by 72 h in rats maintained on either normal diet or on chlordecone diet^a

Age (days)	Diet	Number of rats (n)	% Lethality
20	Normal	10	0
	Chlordecone	20	0
45	Normal	10	0
	Chlordecone	20	25
60	Normal	10	0
	Chlordecone	20	100

^aMale Sprague-Dawley rats were maintained on either normal or 10 ppm chlordecone diet for 15 days. On day 16, a single dose of CCl_4 (100 $\mu\text{l}/\text{kg}$, i.p.) was administered in corn oil to all rats. The animals were observed twice daily for 14 days and cumulative mortality was recorded. No death occurred after 72 h.

Table 2

Effects of colchicine antimetabolism on the lethality of chlordecone + CCl₄ combination treatment in 45 day old rats^a

Group	Treatments	Number of rats (n)	% Lethality
I	ND + CCl ₄	10	0
II	ND + CCl ₄ + CLC	10	0
III	CD + CCl ₄	20	25
IV	CD + CCl ₄ + CLC	20	85

^aThirty-day old male Sprague-Dawley rats were maintained on either normal (ND) or 10 ppm chlordecone (CD) diet for 15 days. On day 16 (45 days old), a single dose of CCl₄ (100 µl/kg, i.p.) was administered in corn oil to all rats. Rats maintained on ND received colchicine (CLC, 1 mg/kg, i.p.) 6 h after administration of CCl₄, whereas those maintained on CD received CLC 30 h after CCl₄. The animals were observed twice daily for 14 days. All deaths occurred within 96 h after administration of CCl₄.

days. The data in Table 1 summarize percent mortality within 72 h after administration of CCl₄. Mortality did not occur in any rats given ND + CCl₄ combination. In CD-pretreated rats, 25 and 100% mortality occurred within 72 h in 45- and 60-day-old rats, respectively. In contrast, no mortality was evident in 20-day-old rats regardless of pretreatment. Antimetabolic intervention with colchicine 30 h after CCl₄ injection to 45-day-old CD rats further increased CCl₄-induced lethality to 85% (Table 2), indicating that cell division is critical for survival through recovery from liver injury.

2.3. Biochemical studies

Hepatic microsomal cytochrome P-450 levels were increased as a function of age (Fig. 2). In 2- and 5-day rats, cytochrome P-450 was significantly lower in comparison to the older age groups. CD pretreatment increased cytochrome P-450. In 20-day CD rats, cytochrome P-450 reached plateau level and significantly higher than the control rats. In control (normal diet, ND) rats, cytochrome P-450 reached plateau level at 35 days after birth (Cai and Mehendale, 1993), indicating that any differences in cytochrome

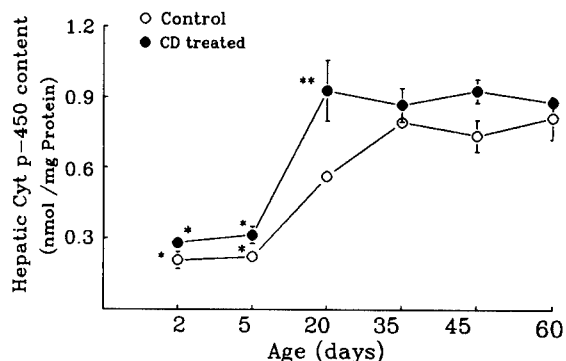


Fig. 2. Hepatic microsomal cytochrome P-450 levels (Cyt P-450) in rats at various ages maintained either on normal diet (ND) or 10 ppm chlordecone (CD) diet for 15 days. Two or three livers were pooled for a single measurement in 2-, or 5-day-old rats. *Denotes significant difference from 20-day-old rats and older rats with the same dietary protocol. **Significant differences between ND and CD 20-day-old rats. Values are means \pm S.E.M. of 4 rats ($P \leq 0.05$). Adapted with permission from Cai and Mehendale (1993).

rome P-450 levels are not responsible for the resiliency of younger rats to hepatotoxicants.

Pretreatment with CD resulted in a significant increase in the proportion of administered ¹⁴CCl₄ expired as ¹⁴CO₂ compared to the ND rats (Fig. 3A). Bioactivation of ¹⁴CCl₄ in 35-day-old CD-treated rats was significantly greater than in 45- and 60-day-old rats as indicated by a greater proportion of the administered ¹⁴CCl₄ expired as ¹⁴CO₂. In vivo metabolism of ¹⁴CCl₄ revealed that about 80% of the administered ¹⁴CCl₄ was expired as unmetabolized parent compound within 6 h regardless of age (Fig. 3B). Sixty-day rats are highly susceptible to CD + CCl₄ lethality whereas none of the 35-day-old pups die from CD + CCl₄ treatment suggesting that differences in bioactivation of CCl₄ are unlikely to be the mechanism (Cai and Mehendale, 1993).

Hepatotoxicity of CCl₄ was assessed by measuring plasma alanine transaminase (ALT) and sorbitol dehydrogenase (SDH) levels during a time course after the injection of CCl₄. It is evident from the ALT data (Fig. 4) that regardless of age, CCl₄ treatment resulted in marginal elevation of ALT levels in rats maintained on

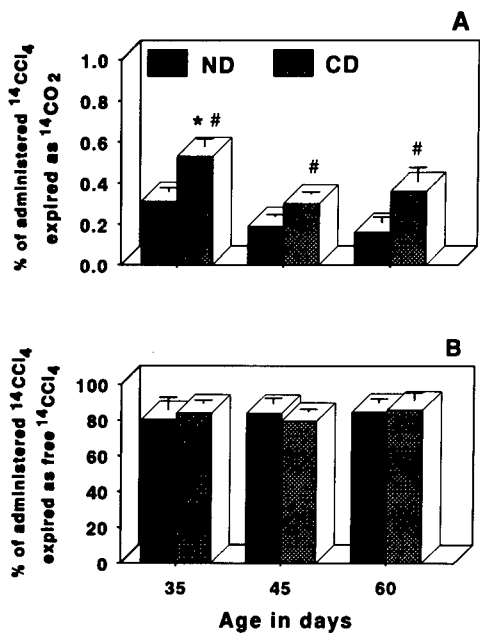


Fig. 3. Measurements of ¹⁴CO₂ (A) and ¹⁴CCl₄ (B) expiration during 6 h at hourly intervals after the administration of ¹⁴CCl₄ to 35-, 45-, and 60-day-old rats maintained either on normal diet (ND) or 10 ppm chlordecone (CD) diet for 15 days. Values are means ± S.E.M. of 4 rats. *Denotes significant difference compared to all age and treatment groups. *Significantly higher values compared to ND rats of the same age ($P \leq 0.05$). Adapted with permission from Cai and Mehendale (1993).

ND. Rats of all age groups promptly recovered from this injury. In contrast, hepatotoxicity was dramatically amplified in rats maintained on CD diet. Significant elevations of plasma enzymes were observed as early as 6 h after the administration of CCl₄. In all age groups, plasma ALT levels peaked between 36 and 48 h after the administration of CCl₄ (Fig. 4). In 60-day-old CD-pretreated rats, plasma enzyme elevations progressed with time until 100% mortality occurred by 72 h. In 45-day-old rats mortality (25%) was observed beginning at 48 h and later time points after CCl₄ administration. As expected, 20-day-old rats receiving CD + CCl₄ did not experience any mortality. After reaching a peak between 36 and 48 h after CCl₄, the plasma enzymes returned to the background level by 72

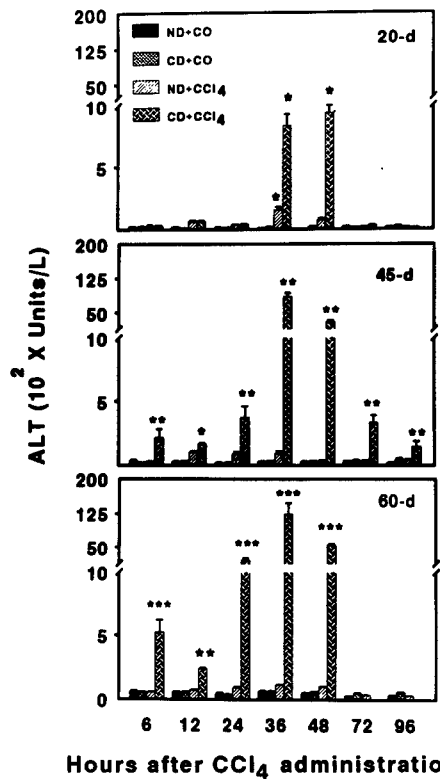


Fig. 4. Plasma alanine transaminase (ALT) concentration during a time-course of 96 h after CCl₄ (100 μl/kg, i.p.) or corn oil (CO) administration to 20-, 45-, and 60-day-old rats maintained on either normal (ND) or 10 ppm chlordecone (CD) diet for 15 days. Values are means ± S.E.M. of 4 rats. *Denotes significant increase in ALT level as compared with the control of same time point. **Significantly higher ALT level than in 20-day-old CD + CCl₄ rats. ***Significantly higher ALT level as compared with that in 20- and 45-day-old CD + CCl₄ rats ($P \leq 0.05$). Adapted with permission from Dalu et al. (1995a).

h, indicating a rapid recovery from CCl₄-inflicted liver injury. Measurement of plasma SDH levels exhibited similar trend as that seen for plasma ALT (data not shown, see Dalu et al., 1995a).

2.4. Age-related differences in hepatic enzymes

There were quantitative differences in plasma enzymes and bilirubin levels among the three age groups, even though actual liver injury as examined under a microscope was very similar among the age groups (Dalu et al., 1995a). These dif-

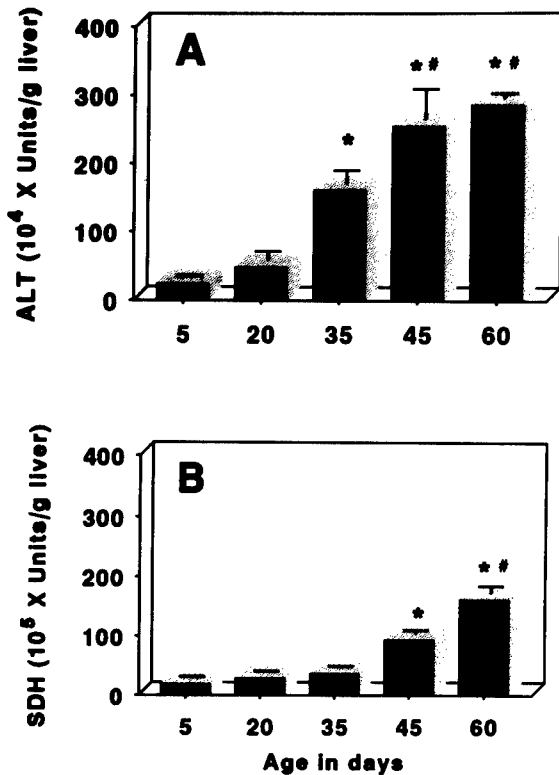


Fig. 5. Hepatic ALT (A) and SDH (B) levels in 5-, 20-, 35-, 45-, and 60-day-old normal male S-D rats expressed per g of liver. Values are means \pm S.E.M. of 4 rats. *Significantly higher levels of hepatic ALT and SDH levels compared to the 5- and 20-day-old rats. **Significantly higher ALT and SDH levels compared to the 35-day-old rats ($P \leq 0.05$).

ferences could be due to: (1) lesser hepatic contents of ALT and SDH in postnatally developing rats than in adult livers; (2) less leakage of the hepatic ALT and SDH in to the blood stream of postnatally developing rats upon toxic liver injury. Measurement of hepatic ALT and SDH in 5, 20, 35, 45, and 60 day normal male Sprague-Dawley rats revealed that 20-day-old rats have less of each hepatic enzyme compared to that in adult rats. Hepatic ALT and SDH contents increased as a function of age and started to level off at 45 days after birth. The results of the study revealed that 20-day-old rats have about 20% of hepatic ALT and SDH in comparison to 60-day-old young adult rats (Fig. 5A, B). Therefore,

lower levels of plasma enzymes in 20-day-old CD rats receiving hepatotoxicants does not mean less injury compared to that seen in adult rats. In 20-day-old rats, lower plasma enzymes simply indicates less availability of hepatic enzymes to leak into the blood stream upon toxic liver injury, rather than indicating lower injury in this age group.

3. Hepatocellular regeneration and tissue repair

3.1. *In vivo* incorporation of [³H]thymidine (³H-T) into hepatic nuclear DNA

³H-T incorporation was measured as an index of S-phase activity of hepatotoxicity. In control rats (0 h time point not treated with CCl₄), there were no significant differences in the incorporation of ³H-T among the three age groups. Likewise, CD-treatment had no significant influence on the incorporation of ³H-T among the three age groups. However, the baseline values in 20-day-old rats were significantly higher than those in older age groups (Fig. 6). Regardless of pretreatment, 20-day-old rats exhibited a higher rate of ³H-T incorporation than the older age groups. The wave of significantly increased ³H-T incorporation began as early as 24 h and continued through 72 h after the administration of CCl₄ in 20-day-old rats. In contrast, in the older rats this response was delayed and diminished. In 45- and 60-day-old ND + CCl₄ rats, ³H-T incorporation increased between 12 and 36 h after CCl₄ treatment and then returned to the base-line, whereas CD + CCl₄-treated rats exhibited slower and diminished ³H-T incorporation, the maximum incorporation being between 48 and 72 h after CCl₄ treatment for 45-day-old rats and 48 h for 60-day-old rats (Fig. 6). The 75% survival in 45-day-old CD rats is presumably due to a very strong response in tissue repair activity particularly between 48 and 72 h after the administration of CCl₄. The delayed and diminished ³H-T incorporation in adult rats (60-day-old) is a reflection of the suppressed S-phase stimulation and tissue repair, presumably leading to a loss of restraint on the progression of liver injury culminating in 100% mortality.

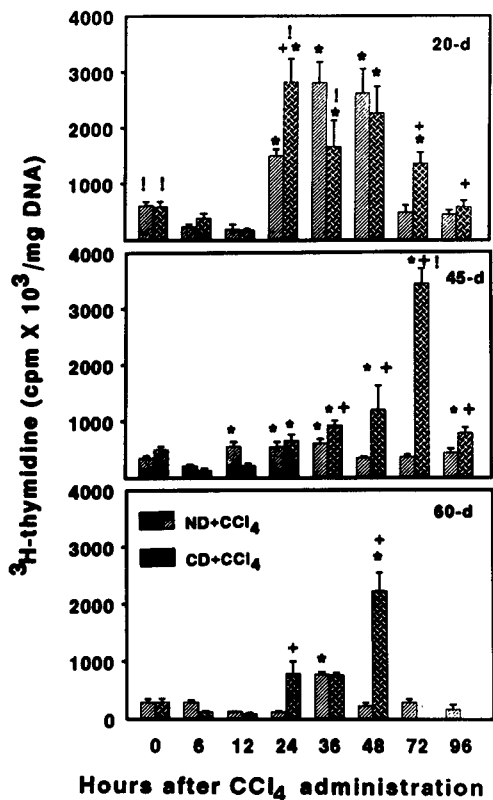


Fig. 6. [^3H]thymidine incorporation into hepatic nuclear DNA during a time course after CCl_4 ($100 \mu\text{l/kg}$, i.p.) administration to 20-, 45-, and 60-day-old rats maintained on either normal (ND) or 10 ppm chlordecone (CD) diet for 15 days. Values are means \pm S.E.M. of 4 rats. *Denotes significantly higher value as compared with the respective control group (0 h time point). †Significantly higher value as compared with ND + CCl_4 rats at the corresponding time period. ‡Significantly higher value as compared with corresponding pretreatment in 60-day-old CD + CCl_4 rats ($P \leq 0.05$). Adapted with permission from Dalu et al. (1995a).

3.2. Proliferating cell nuclear antigen (PCNA) study

PCNA immunohistochemical staining procedure was used to confirm the $^3\text{H-T}$ incorporation study. This technique makes it possible to identify cells in different phases of the cell cycle (Figs. 7-9). The results of PCNA study were concordant with the hepatocellular proliferative activity assessed by $^3\text{H-T}$ incorporation study. Normally, most cells in the liver are in the resting or quiescent (G_0) phase and a relatively small

number of cells (4-6%) are in other phases. In older control rats (45- and 60-day-old), almost all the cells were quiescent (Figs. 8A,B; 9A,B), whereas in 20-day-old rats, relatively greater number of cells were in G_1 and S-phase of the cell cycle (Fig. 7A, B) regardless of pretreatment, indicative of ongoing liver growth.

After the administration of CCl_4 ($100 \mu\text{l/kg}$), hepatocytes progressed to G_1 , S, G_2 , or M phase of cell cycle by 24 h after CCl_4 . A maximum number of cells were seen in S-phase between 36 and 48 h after CCl_4 by this method regardless of pretreatment (Fig. 7E, F), consistent with the peak of $^3\text{H-T}$ incorporation during the same time points (Fig. 6). Population of cells in G_2 and M phase also increased during the same time frame (Fig. 7E, F). By 96 h, the proliferative activity returned close to the background level in both ND + CCl_4 and CD + CCl_4 treatment groups.

In contrast to CCl_4 -induced hepatocyte proliferative activity in 20-day-old rats, this activity was delayed and diminished in 45- and 60-day-old rats. In 45-day-old ND rats a maximum number of cells were seen in G_1 and S-phase 36 h after CCl_4 (Fig. 8E), whereas in CD-treated rats this did not occur till 72 h after CCl_4 (Fig. 8F). Although a significant number of cells progressed to S, G_2 and M phase in 60-day-old CD rats at 48 h after CCl_4 (Fig. 9D), the overall number of divided cells were insufficient to replace the progressively increasing number of dead cells, thereby leading to 100% mortality by 72 h. These findings demonstrate that liver injury progresses if timely tissue repair is not stimulated, suggesting the dynamic relationship between progression of injury and the opposing tissue repair response.

4. Molecular signals of cell division and tissue repair

Substantial experimental data indicate that hepatocellular regeneration is subject to regulation by growth factors such as transforming growth factors- α (TGF- α) and TGF- β (Fausto and Mead, 1989; Armendariz-Borunda et al., 1990; Michalopoulos, 1990). One other event thought to be essential for tissue regeneration is

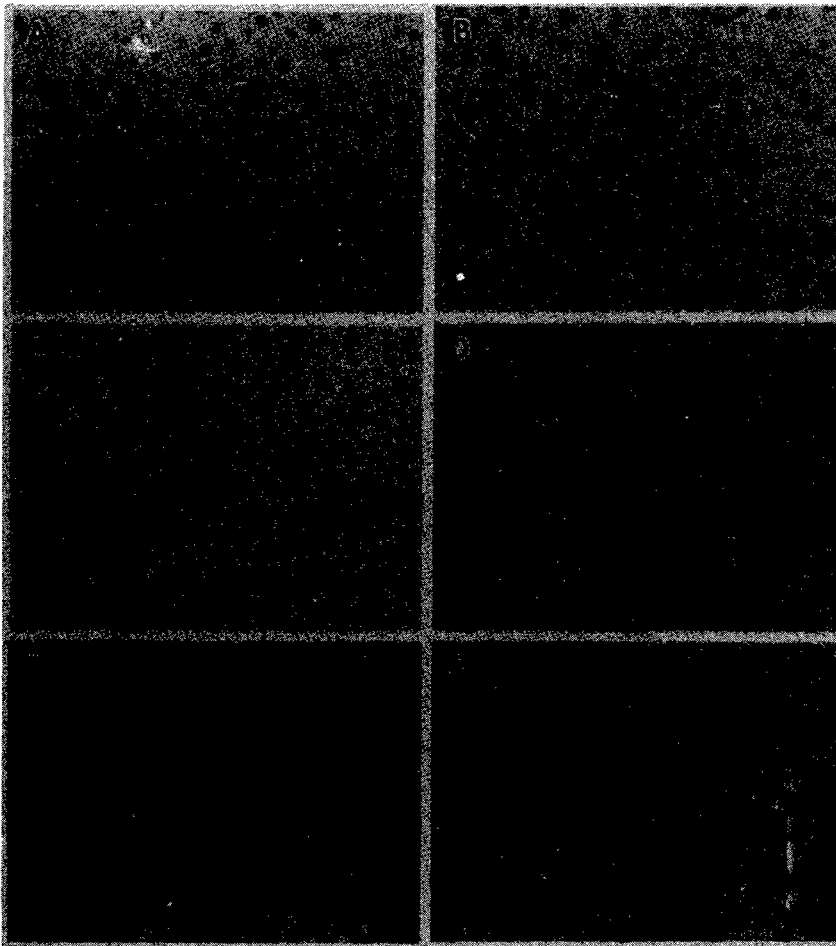


Fig. 7. Proliferating cell nuclear antigen (PCNA) immunohistochemical analyses in 20-day-old rats. (A) Control for ND. (B) Control for CD. (C) and (D) Time at which significant population of cells progressed to S-phase (24 h) for ND + CCl₄ and CD + CCl₄ groups, respectively. (E) and (F) Time at which maximum number of cells were seen in S, G₂, and M phase (48 h) for ND + CCl₄ and CD + CCl₄ groups, respectively. G₀, cells with no staining; G₁, cells with light brown nuclear staining; S, cells with deep brown nuclear staining; G₂, cells with or without speckled nuclear staining and brown cytoplasmic staining; and M, cells with diffused cytoplasmic staining and with deep blue chromosomal staining.

activation of specific early (*c-fos*) and late (*H-ras*) proto-oncogenes, which are known to influence the division competency of surrounding cells through "cell priming" (Rollins and Stiles, 1988; Fausto and Mead, 1989). Expressions of TGF- α as well as early (*c-fos*) and late (*H-ras*) proto-oncogenes have been shown to be increased in age- and time-dependent manner after the administration of a low dose of CCl₄ (Dalu et al.,

1995b). The timing of the TGF- α mRNA expression coincided with the major wave of CCl₄-induced DNA synthesis. It could be suggested that the efficient hepatocellular regenerative and tissue repair activities in 20-day-old rats is due to higher levels of TGF- α as well as differences in the expressions of early and late proto-oncogenes stimulated by CCl₄. Therefore, the possibility that the age-related differences observed in CCl₄

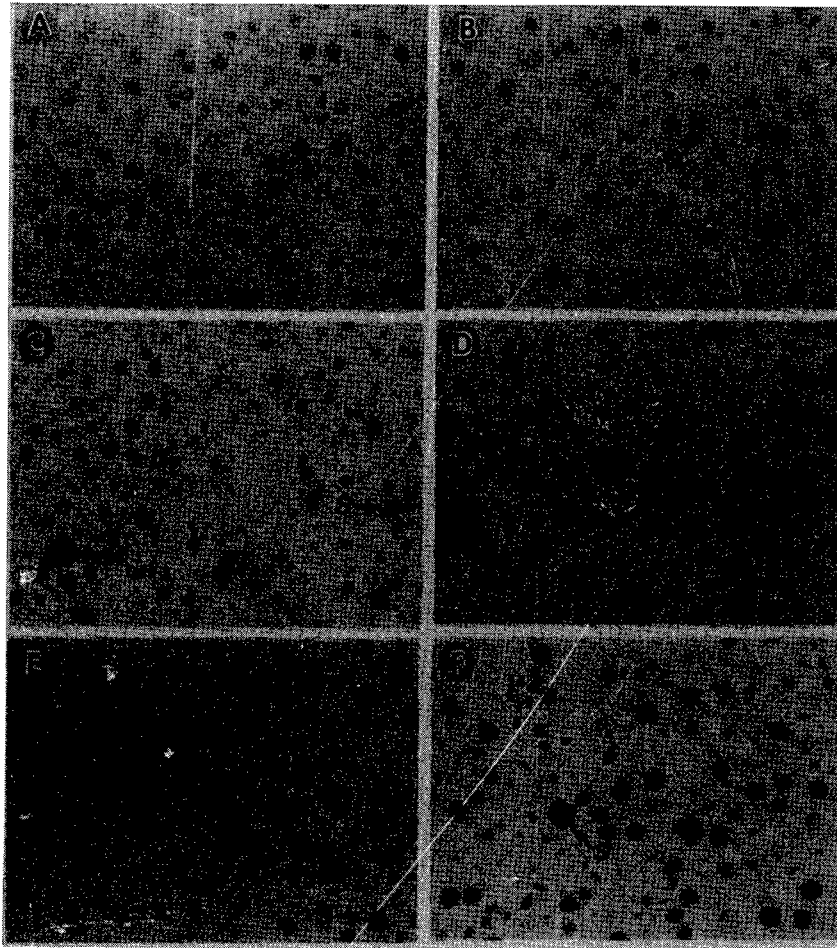


Fig. 8. Proliferating cell nuclear antigen (PCNA) immunohistochemical analyses in 45-day-old rats. (A) Control for ND. (B) Control for CD. (C) and (D) Time at which significant population of cells progressed to S-phase for ND + CCl₄ (12 h) and CD + CCl₄ (36 h) groups, respectively. (E) and (F) Time at which maximum number of cells were seen in S, G₂, and M phase for ND + CCl₄ (36 h) and CD + CCl₄ (72 h) groups, respectively. Identification of the cells is as described under Fig. 7.

hepatotoxicity potentiated by CD are due to differences in the expressions of early proto-oncogenes needs to be further investigated. In addition to ongoing cell division, our findings also suggest that quicker and comparatively higher cell division occurs during early postnatal development upon challenge by a toxicant. This may be due to either quicker stimulation of TGF- α expression, or inhibition of TGF- β expression, upon toxicant challenge. The role of TGF- α , TGF- β , and the early proto-oncogenes in age-

related hepatocellular regeneration will be of continued research interest and worthy of further investigation.

5. Summary and conclusions

It is well known that rats during postnatal development are resilient to a wide variety of structurally and mechanistically dissimilar hepatotoxicants (Dawkins, 1963; Cagen and Klaassen, 1979; Green et al., 1984; Rikans, 1989; Abdul-

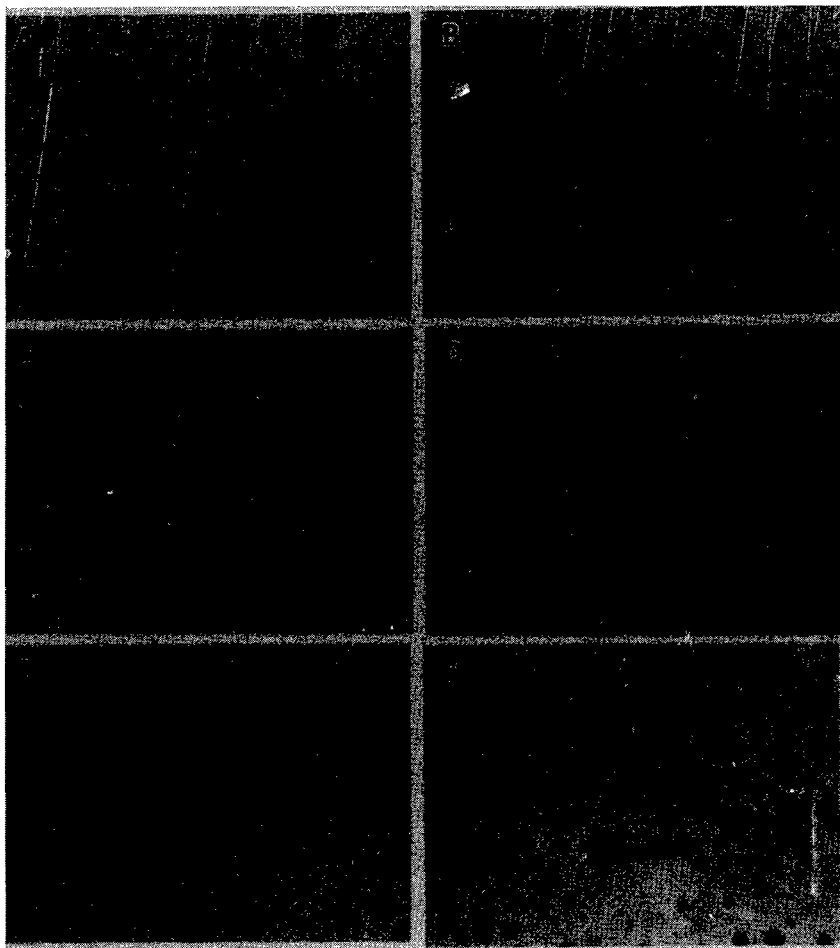


Fig. 9. Proliferating cell nuclear antigen (PCNA) immunohistochemical analyses in 60-day-old rats. (A) Control for ND. (B) Control for CD. (C) and (D) Time at which significant population of cells progressed to S-phase for ND + CCl₄ (12 h) and CD + CCl₄ (24 h) groups, respectively. (E) and (F) Time at which maximum number of cells were seen in S, G₂, and M phase for ND + CCl₄ (24 h) and CD + CCl₄ (48 h) groups, respectively. Identification of the cells is as described under Fig. 7.

Hussain and Mehendale, 1992; Cai and Mehendale, 1993; Dalu et al., 1995a). Studies by Dawkins (1963) and Cagen and Klaassen (1979) indicate that newborns are resilient to the toxic effect of CCl₄. In the same studies, 4-, 7-, 10-, 14-, and 21-day-old rats were reported to be as sensitive as adult rats when high doses are used (1–2 ml/kg). It is known that cell division and tissue repair mechanisms are inhibited at high doses of toxic chemicals leading to progression of injury (Kodavanti et al., 1989b; Rao and Mehendale, 1991; Rao et al., 1994; Mangipudy et al., 1995).

Studies by Cai and Mehendale (1993) indicate that younger rats are resilient not only to CCl₄ lethality, but also to the lethal combination of CD + CCl₄ known to cause 100% mortality in adult rats (Kligensmith and Mehendale, 1982; Agarwal and Mehendale, 1983; Soni and Mehendale, 1993).

At least two major mechanisms might explain the age-related differences in CCl₄-induced hepatotoxicity and lethality. One possible mechanism is the lower cytochrome P-450 in the younger rats giving rise to the lower bioactivation of CCl₄

and correspondingly lower lipid peroxidation compared to that in the adult rats. Furthermore, the resiliency of CD + CCl₄ treated younger rats might be due to lower cytochrome P-450 induction by CD compared to that in the adult rats, leading to proportionally lower bioactivation of CCl₄ to form the free radicals required for the initiation of liver injury. However, although 35-, 45-, and 60-day-old rats have comparable cytochrome P-450 levels (Fig. 2), the 35-day-old CD rats do not experience CCl₄-induced lethality as 45- and 60-day-old CD rats (Cai and Mehendale, 1993). Furthermore, *in vivo* metabolism of CCl₄ (Fig. 3A, B) in terms of ¹⁴CO₂ production derived from ¹⁴CCl₄ and ¹⁴CCl₄ metabolites bound to hepatic tissue were not significantly different between 35-, 45-, and 60-day-old CD rats. Therefore, differences in cytochrome P-450 levels or bioactivation-dependent mechanisms do not provide adequate explanation for the resiliency of rats during postnatal development to CCl₄ or to CD + CCl₄ hepatotoxicity and lethality (Cai and Mehendale, 1993).

The second possible mechanism that may explain the resiliency of younger rats to CD + CCl₄ hepatotoxicity and lethality is the ongoing liver growth and toxicant-stimulated tissue repair mechanisms upon toxic liver injury. The overall findings indicate that the resiliency observed in the younger rats is associated more with ongoing liver growth and additional stimulation of hepatocellular regeneration and tissue repair mechanisms.

Upon exposure to a single low dose of CCl₄ (100 μl/kg), transient liver injury occurs regardless of age between 6 and 36 h as evidenced by elevation of plasma ALT (Figs. 4) and supported by histopathology (Dalu et al., 1995a). During this period of injury ongoing as well as stimulated hepatocellular proliferation and tissue repair activities were evident until 48 h as evidenced by ³H-T incorporation data (Fig. 6) and PCNA study (Figs. 7-9). This response was much stronger in 20-day rats in comparison to that observed in 45- and 60-day-old rats. Consequently, progression of tissue injury in 20-day-old rats was restrained, leading to full recovery. In CD rats, CCl₄-induced hepatotoxicity (Fig. 4)

and lethality (Table 1) were highly amplified by 25 and 100% in 45- and 60-day-old rats, respectively. No hepatocellular regenerative and tissue repair activity was evident in 60-day-old CD rats until 48 h. S-phase stimulation observed at 48 h in 60-day CD rats (Figs. 6 and 9F) was too little and too late to restrain the progression of liver injury. Consequently, unrestrained progression of liver injury led to liver failure and 100% mortality in 60-day-old rats (Soni and Mehendale, 1993; Dalu et al., 1995a). Despite similar liver injury, 20- and 45-day-old rats differ in that this injury becomes progressive in 45-day-old rats, leading to 25% mortality, whereas 20-day-old rats escape death entirely. In 45-day CD rats, hepatocellular regeneration and tissue repair increased significantly between 36 and 72 h after the administration of CCl₄, as revealed by ³H-T incorporation and PCNA studies. In contrast to the effect in 45- and 60-day-old CD-rats, CD treatment did not affect ongoing liver growth or CCl₄-stimulated hepatocellular regeneration and tissue repair in 20-day-old rats. In 20-day-old rats, significant hepatocellular regeneration and tissue repair were evident in both treatment groups (ND + CCl₄ and CD + CCl₄) at 24 h, continuing through injection of CCl₄, as indicated by ³H-T incorporation and PCNA studies (Figs. 6 and 7). At 48 h after the administration of CCl₄, a large number of cells in 20-day-old rats progress to G₂ and M phase regardless of pretreatment (Fig. 7E,F). These findings suggest that the presence or absence of ongoing liver growth and additional prompt stimulation of hepatocellular regeneration and tissue repair play a critical role in regression or progression of injury, respectively (Dalu et al., 1995a).

The extent of CCl₄-induced liver injury in all CD-treated rats was comparable regardless of age. The timely and substantial S-phase stimulation and cell replication throughout the injury phase played a key role in full and speedy recovery of 20-day-old rats. Therefore, ongoing liver growth and differences in the promptness of the hepatocellular regenerative response may be of pivotal importance in explaining the age-related differences in hepatotoxicity. Altogether, the findings indicate that age-related differences in CD-

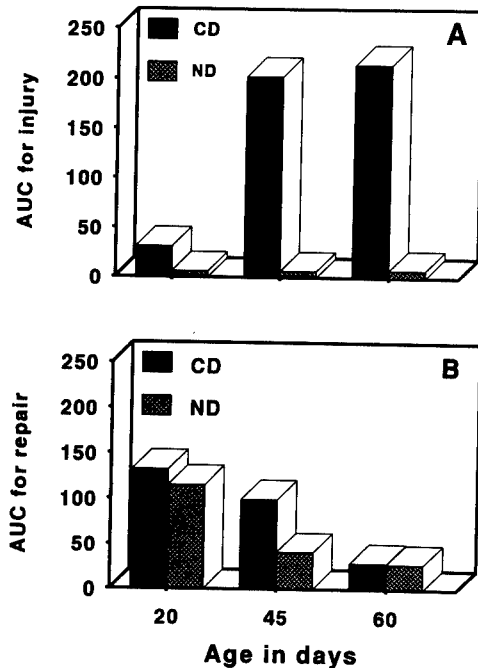


Fig. 10. Areas under the curves (AUC) for CCl₄-induced tissue injury (A) and tissue repair (B) during a time course of 0-96 h after CCl₄ (100 μ l/kg, i.p.) administration to 20-, 45-, and 60-day-old rats maintained on either normal (ND) or 10 ppm chlordecone (CD) diet for 15 days. Liver injury (Panel A) was assessed by measuring plasma ALT activity (Units/l) and liver tissue repair (Panel B) was assessed by measuring stimulation of S-phase synthesis (³H-T incorporation in counts/min/mg DNA) during 0 to 96 h time course. Areas under the two respective curves were calculated using BASIC statistical program and plotted for each age group. It should be noted that while AUC simplifies the overall estimations, it takes away any temporal (e.g. early onset or delay) relationships.

potentiated CCl₄ hepatotoxicity and lethality are associated with the balance between two opposite biological events occurring simultaneously. CCl₄-induced liver injury and tissue repair (Fig. 10), as demonstrated by areas under the curves (AUC) for ALT and ³H-T incorporation, respectively, illustrate this point. For example, the AUC for CCl₄-induced liver injury in 20-day-old CD-rats is one-tenth of that observed in 45- and 60-day-old rats (Fig. 10A). The AUC for tissue repair induced by CCl₄ in 20-day-old CD-rats is about 5-fold higher than that observed in 60-day-

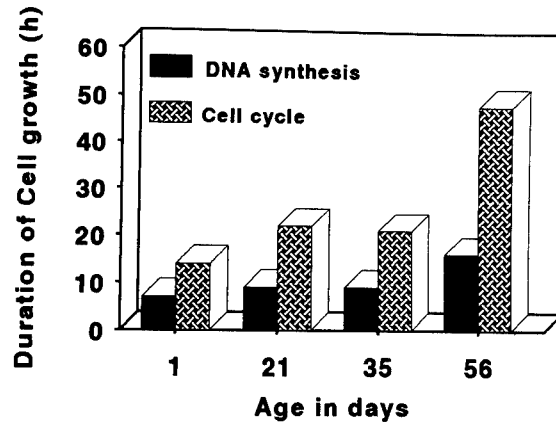


Fig. 11. Effects of age on the duration of liver tissue growth and renewal. Time required for completion of S-phase DNA synthesis and for completion of cell cycle in 1-, 21-, 35-, and 56-day-old rats. In younger rats, both DNA synthesis and cell cycle take about half the time in contrast to duration observed in adult (56-day) rats. Adapted with permission from Altman and Dittmer (1972).

old CD-rats (Fig. 10B). The AUC for tissue repair in 45-day-old rats is similar to that calculated for 20-day-old rats, correlating with the 75% survival of 45-day-old CD rats receiving CCl₄.

Why are rats so efficient in tissue repair during early postnatal development? One reason is the much higher efficiency of DNA synthesis, as illustrated by the shorter duration required for S-phase and the cell cycle (Altman and Dittmer, 1972). In younger rats (21-day), both DNA synthesis and the cell cycle completion require about half the time as long as in 56-day rats (Fig. 11). Therefore, the efficiency in tissue repair in postnatally developing rat livers appears to be responsible for timely and substantial tissue repair response, allowing the regression of liver injury inflicted by CCl₄.

In summary, the findings of the present study indicate that postnatally developing rats are resilient to the hepatotoxic and lethal effects of CD + CCl₄ combination. This remarkable resiliency appears to be related to ongoing liver growth and the ability to stimulate additional cell division and tissue repair promptly. The findings of the current study provide significant insight in

understanding the mechanisms in control of cell division stimulated during toxic injury. Additional insights into the mechanisms regulating the quiescence of adult liver cells and plasticity of growing livers are likely to open up new avenues of therapy, drug development, and refined assessment of age-related risk to public health from exposure to toxic chemicals.

These findings are of particular interest in risk assessment. A significant flaw in the current risk assessment procedures is the sole reliance on the mechanisms responsible for infliction or initiation of injury (stage I) in projecting the outcome of that injury over time. Substantial evidence is now available to suggest that often injury is initiated, whether that injury progresses or regresses depends vastly on the biological mechanisms including the dynamics of cell birth and cell death (Mehendale, 1991, 1994, 1995; Rao et al., 1994; Chanda and Mehendale, 1995; Mangipudy et al., 1995). Including appropriate measures of mechanisms responsible for initiating cell death as well as increases of the compensatory biological mechanisms of cell birth that bear a continuous dynamic relationship with cell death by opposing the progression of tissue injury should be included to fine-tune risk assessment procedures. Recent studies in which dose-response relationships have been studied for injury and tissue repair as opposing, but simultaneous dynamic responses have yielded promising results (Rao et al., 1994; Mangipudy et al., 1995). Because age-related susceptibility to toxic chemicals may also be a function of the degree and promptness with which tissue repair is stimulated (Abdul-Hussain and Mehendale, 1991, 1992; Cai and Mehendale, 1993; Dalu et al., 1995a,b), risk assessment paradigms which include dynamic measures of cell death and cell birth are likely to address the age-related susceptibility issues as well.

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Toxicology 111 (1996) 43–65

TOXICOLOGY

Phenotypic variation in xenobiotic metabolism and adverse environmental response: focus on sulfur-dependent detoxification pathways

Stephen A. McFadden

Independent Research Advocates, 5521 Greenville Ave., no. 104-608, Dallas, TX 75206, USA

Abstract

Proper bodily response to environmental toxicants presumably requires proper function of the xenobiotic (foreign chemical) detoxification pathways. Links between phenotypic variations in xenobiotic metabolism and adverse environmental response have long been sought. Metabolism of the drug *S*-carboxymethyl-*L*-cysteine (SCMC) is polymorphous in the population, having a bimodal distribution of metabolites, 2.5% of the general population are thought to be nonmetabolizers. The researchers developing this data feel this implies a polymorphism in sulfoxidation of the amino acid cysteine to sulfate. While this interpretation is somewhat controversial, these metabolic differences reflected may have significant effects. Additionally, a significant number of individuals with environmental intolerance or chronic disease have impaired sulfation of phenolic xenobiotics. This impairment is demonstrated with the probe drug acetaminophen and is presumably due to starvation of the sulfotransferases for sulfate substrate. Reduced metabolism of SCMC has been found with increased frequency in individuals with several degenerative neurological and immunological conditions and drug intolerances, including Alzheimer's disease, Parkinson's disease, motor neuron disease, rheumatoid arthritis, and delayed food sensitivity. Impaired sulfation has been found in many of these conditions, and preliminary data suggests that it may be important in multiple chemical sensitivities and diet responsive autism. In addition, impaired sulfation may be relevant to intolerance of phenol, tyramine, and phenolic food constituents, and it may be a factor in the success of the Feingold diet. These studies indicate the need for the development of genetic and functional tests of xenobiotic metabolism as tools for further research in epidemiology and risk assessment.

Keywords: Xenobiotic metabolism; Sulfur-dependent detoxification pathways; Risk assessment; Sulfoxidation; Sulfation; Idiopathic environmental intolerance

1. Genetic polymorphisms in xenobiotic metabolism and environmental susceptibility

Sensitive human subpopulations have been considered in a number of meetings and publications over the past two decades. These include works on susceptibility to medical and environmental exposures, works on ecogenetics—the

genetics of environmental susceptibility, works on pharmacogenetics—the genetics of drug metabolism, works on psychopharmacogenetics—the relationship of genetics to psychopharmacology, works on a specific polymorphism, and works on biomarkers—the methods used to test for toxic exposure, susceptibility, and effect (Eleftheriou, 1975; Mendlewicz, 1975; Vogel et al.,

1978; Ciba Foundation, 1980; Calabrese, 1984; Omenn and Gelboin, 1984; Weber, 1987; Woodhead et al., 1988; Bartsch et al., 1992; Kalow, 1992; Price-Evans, 1993; Woods and Sever, 1993; IBC USA Conferences Inc., 1995). Organizations involved in this research include the Ciba Foundation, Cold Spring Harbor Laboratory, Brookhaven National Laboratory, National Institute of Environmental Health Sciences (NIEHS), and Battelle Pacific Northwest Laboratories.

The journals *Xenobiotica* and *Pharmacogenetics* are also concerned with the subject of sensitive human populations. In 1992, NIEHS established an Environmental Health Sciences Center devoted to this topic, the Center for Environmental Genetics (CEG) of the University of Cincinnati Medical Center, under the direction of D.W. Nebert (National Institute for Environmental Health Sciences, 1994; University of Cincinnati, 1995). The subject of sensitive human subpopulations is becoming of increasing interest to industry, government, and the military.

Proper bodily response to environmental toxicants presumably requires proper function of the xenobiotic (foreign chemical) detoxification pathways. Of those factors contributing to variability in human response to toxicants, it can be expected that genetic and acquired variations in the metabolism and excretion of xenobiotics constitute major influences.

This paper focuses on variations in the metabolism of sulfur compounds seen in some members of the population, which may produce or reflect disturbances in several xenobiotic detoxification pathways, in addition to possibly having direct toxic effects. Specifically, the metabolism of the drug *S*-carboxymethyl-L-cysteine (SCMC) is polymorphous in the population, having a bimodal population distribution of metabolism rate; in addition, about 2.5% of the general population are thought to be nonmetabolizers—a group about which this paper is particularly concerned. The researchers developing this data interpret it as implying a polymorphism in the sulfoxidation of the amino acid cysteine to sulfate, which may lead to elevated thiols and reduced sulfate levels.

Sulfation is a detoxification pathway for phenolics which is dependent upon a depletable

supply of sulfate substrate, presumably produced by the sulfoxidation of cysteine. Impaired sulfation, for whatever reason, can be expected to increase susceptibility to a number of xenobiotics, in addition to altering the metabolism of some endogenous mediators and biocomponents such as steroids, catecholamine neurotransmitters, and bile acids. Reduced metabolism of SCMC and impaired sulfation appear with increased frequency in clinical studies of those with several forms of disease or adverse environmental response.

2. Introduction to xenobiotic metabolism

The basics of xenobiotic metabolism are reviewed in "Casarett & Doull's Toxicology: The Basic Science of Poisons" (Sipes and Gandolfi, 1986).

While most water soluble compounds that are not usually of normal xenobiotic metabolic importance are readily absorbed from the digestive tract and excreted via the kidneys in urine, fat soluble xenobiotics are typically absorbed from the digestive tract, are oxidized to intermediates that may be highly reactive, are conjugated to increase solubility, and are then excreted either by the kidneys in urine, or by the liver in bile back into the digestive tract.

Specifically, while most polar water soluble compounds for which there is no specific renal conservation mechanism are readily excreted in urine, elimination of renally conserved, nonpolar fat soluble compounds tends to be more troublesome to the body, requiring metabolism for elimination (Table 1). "Phase I" activation reactions typically activate such nonpolar xenobiotics to make them more reactive, for instance, by oxidation by the cytochrome P450 family of enzymes. "Phase II" conjugation reactions take compounds with an active functional group, including compounds activated in Phase I reactions, and add an endogenous substrate to that group to make the compounds more soluble and/or reduce their toxicity. Phase II conjugates include glucuronic acid, sulfate, glutathione, acetyl, glycine, and methyl groups. Some have included as a classification "Phase III" elimination path-

Table 1
Some important human xenobiotic detoxification pathways (after Sipes and Gandolphi, 1986)

Pathway (enzyme or conjugate)	Typical xenobiotic substrates
(1) Phase I "activation" reactions (e.g., of nonpolar "fat soluble" compounds) Cytochrome P450 family	C, N, or S oxidation/hydroxylation of aromatic and aliphatic hydrocarbons
(2) Phase II "conjugation" reactions (e.g., of active functional groups) Glucuronic acid Sulfate Glutathione and cysteine Acetate Glycine Methyl	Hydroxyl, carboxyl, and amine groups Phenolics (aromatic hydroxyl groups), catecholamines, and steroids Aromatics, metal ions Amines (including many sulfa drugs) Aromatic acids Aliphatic thiols, hydrogen sulfide
(3) Phase III "elimination" pathways Renal excretion Biliary excretion Sebaceous excretion Lactation	Polar, low molecular weight Polar, intermediate molecular weight (note that some conjugates may be deconjugated and reabsorbed via enterohepatic recirculation) Nonpolar Many

ways, to take into account the dynamics of elimination. With the biliary excretion of glucuronides, this may involve a process of deconjugation within the digestive tract and subsequent reabsorption known as enterohepatic recirculation.

Not all xenobiotic metabolism reactions ultimately reduce toxicity. Some may activate procarcinogens, for instance, the Phase I cytochrome P450 oxidation of benzo[*a*]pyrene, a polycyclic aromatic combustion product, and the Phase II *N*-acetylation of benzidine, an aromatic diamine structurally related to the aniline dyes.

A number of xenobiotic metabolism pathways are known to be polymorphous in humans (Table 2). An example of a well-known genetic polymorphism in xenobiotic metabolism is the *N*-acetylation of amines, required to allow amines otherwise renally conserved to be excreted (see Weber, 1987). *N*-acetylation varies roughly a factor of 3 between fast and slow acetylators, with a population range of about ten-fold. Approximately 50% of American Caucasians and Blacks are fast acetylators, and 50% slow, while Japanese are 90% fast (see Karim et

al., 1981; Weber and Hein, 1985; McKusick, 1988). The *N*-acetylation polymorphism was relevant to the occupational risks of bladder cancer from exposure to the aniline dyes during the 1800s, and the pharmacokinetics of the early sulfonamide antibiotics developed in the 1950s. It may also be relevant to more modern occupational hazards, given the use of aromatic diamines as epoxy hardeners in advanced composite materials, such as used by the aerospace industry (Larson and Scheide, 1989; Kantz, 1989; Schwartz, 1989).

The study of genetic polymorphisms in xenobiotic metabolism, however, is still young. Calabrese (1984) reviewed approximately 50 conditions involving susceptibility to environmental agents and concluded that, while there are excellent theoretical foundations for ecogenetics, only one condition had a demonstrated causal history of enhancing one's susceptibility to industrial pollutants: glucose-6-phosphate dehydrogenase (G6PD) deficiency resulting in hemolytic anemia under oxidant stress conditions (such as exposure to TNT or naphthalene).

Table 2
Selected genetic polymorphisms in xenobiotic metabolism (after Calabrese, 1984; Waring and Emery, 1993)

Pathway	Example and relevance	Frequency	Genetics	Reference
Cytochrome P450	Type II-D6 hydroxylation of debrisoquine and more than	2-10% of population (depending on ethnic	Chromosome 22 at CYP2D6	Calabrese, 1984; McKusick, 1988
N-Acetylation of hydrazine and arylamines	Drugs: isoniazid, dapsone, and many sulfonamides; Carcinogens: benzidine and many aniline dyes	50% fast/50% slow in U.S. Caucasian and Black population; 90% fast/10% slow in Japanese	Enzymes polymorphous	Weber and Hein 1985, Weber 1987
Glucuronidation	Relevant to the detoxification of PCBs and a conjugation pathway for bilibubin, the most common detoxification pathway; deficient in infants and Gilbert's syndrome	Gilbert's syndrome in approx. 6% of U.S. population	Genetic component	Calabrese 1984
Sulfation	Steroids and phenolics (aromatic hydroxyl groups) including many catecholamine neurotransmitters and their analogs, acts as a first pass barrier to the absorption of many dietary phenolics	TS-PST (PST-P): allele frequency .20 high, .80 low TL-PST (PST-M): allele frequency .08 high, 0.92 low	TS-PST (PST-P): 81% heritable TL-PST (PST-M) 77% heritable Function dependent on sulfate from the sulfoxidation of cysteine	Calabrese 1984 Healfield et al. 1990
Methylation	Thiols and hydrogen sulfide; thiopurine methyltransferase (TPMT) (methylates aromatic thiols such as thiopurines) and aliphatic methyltransferase (ATMT) (methylates aliphatic thiols such as D-penicillamine)		Both are believed to be polymorphous and under genetic control	Waring and Emery 1993

3. Introduction to basic human sulfur metabolism

Most sulfur is absorbed by the body in organic form as sulfur amino acids containing thiol (R-SH), thioether (R-S-R), or disulfide (R-S-S-R) groups (Fig. 1, Table 3). These are for the most part eventually oxidized and excreted as inorganic sulfate (SO_4^{2-}) (Rosenberg and Scriver, 1974; Cooper, 1983; Griffith, 1987). Only a small amount of dietary sulfate is absorbed (Florin et al., 1991). This lack of absorption is thought to be due to the tendency of inorganic sulfates to form strong acids and insoluble salts. The sulfoxidation of the amino acid cysteine by the enzyme cysteine dioxygenase is thus thought to be a gateway step in sulfur metabolism, between the absorption of organic thiols and the excretion of inorganic sulfate (Rosenberg and Scriver, 1974). However, an alternative pathway

involving 3-mercaptopyruvate is important in metabolizing some sulfur compounds such as D-cysteine (Cooper, 1983). It has been suggested that hepatic glutathione levels may reflect the metabolic pool of sulfur amino acids (e.g. thiols), while urinary taurine excretion may reflect their catabolism (to sulfates (Hosokawa et al., 1988).

The enzyme cysteine dioxygenase oxidizes cysteine to cysteine sulfinic acid, which may be metabolized to either taurine, or to sulfate and pyruvate. Cysteine dioxygenase is an iron-containing enzyme located in liver cytosol (Yamaguchi et al., 1978; Waring et al., 1986; Yamaguchi and Hosokawa, 1987; Mitchell and Waring, 1989b). Cysteine dioxygenase activity is diurnal, with maximum activity during daytime (Waring and Emery, 1993). The human and Norway rat liver cDNA have been isolated and characterized (Hosokawa et al., 1990; McCann et

Table 3
Sulfur compounds of physiological and pharmaceutical interest

1. Physiological sulfur compounds: amino acids, intermediates, and metabolites

1.1. Thiols, thioethers, and disulfides

Methionine	$\text{NH}_2\text{-CH}(\text{COOH})\text{-CH}_2\text{-CH}_2\text{-S-CH}_3$
Homocysteine	$\text{NH}_2\text{-CH}(\text{COOH})\text{-CH}_2\text{-CH}_2\text{-SH}$
Cystathionine	$\text{NH}_2\text{-CH}(\text{COOH})\text{-CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}(\text{COOH})\text{-NH}_2$
Cysteine	$\text{NH}_2\text{-CH}(\text{COOH})\text{-CH}_2\text{-SH}$
Cystine	$\text{NH}_2\text{-CH}(\text{COOH})\text{-CH}_2\text{-S-S-CH}_2\text{-CH}(\text{COOH})\text{-NH}_2$
Glutathione	Glutamyl-cysteinyl-glycine tripeptide
Cysteamine	$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-SH}$
Cystamine	$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-S-S-CH}_2\text{-CH}_2\text{-NH}_2$
3-Mercapto pyruvate	$\text{COOH-C(=O)-CH}_2\text{-SH}$

1.2. Sulfinates, sulfonates, sulfite, and sulfate

Cysteine sulfinic acid	$\text{NH}_2\text{-CH}(\text{COOH})\text{-CH}_2\text{-SO}_2\text{H}$
Hypotaurine	$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-SO}_2\text{H}$
Taurine	$\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-SO}_3\text{H}$
B-Sulfinyl pyruvate	$\text{COOH-C(=O)-CH}_2\text{-SO}_2\text{H}$
Sulfite	SO_3
Sulfate	SO_4

2. Pharmacological sulfur compounds: cysteine analogs and analog metabolites

2.1. Cysteine analogs

S-Carboxymethyl-L-cysteine (SCMC)	$\text{NH}_2\text{-CH}(\text{COOH})\text{-CH}_2\text{-S-CH}_2\text{-COOH}$
Carboxymethylthio-L-cysteine (CMTC)	$\text{NH}_2\text{-CH}(\text{COOH})\text{-CH}_2\text{-S-S-CH}_2\text{-COOH}$
D-Penicillamine	$\text{NH}_2\text{-CH}(\text{COOH})\text{-C}(\text{CH}_3)_2\text{-SH}$
Sodium aurothiomalate	$\text{CH}_2(\text{Na}^+\text{COO}^-)\text{-CH}(\text{Na}^+\text{COO}^-)\text{-S-Au}$

2.2. Cysteine analog metabolites

Thioglycolic acid	$\text{COOH-CH}_2\text{-SH}$
Thiodiglycolic acid	$\text{COOH-CH}_2\text{-S-CH}_2\text{-COOH}$

al., 1994). In rat studies, hepatic cysteine dioxygenase activity is regulated by dietary protein content and the intake of dietary sulfur amino acids; and it may be induced by injection of cysteine or methionine in the presence of corticosteroids. (Hosokawa et al., 1988). Cysteine may also be oxidized by a NAD-dependent enzyme of lesser capacity that exists in other tissues, including the brain (Yamaguchi et al., 1978, 1985). Brain cysteine dioxygenase is deficient in the globus pallidus in Hallervorden-Spatz disease (Perry et al., 1985).

There are two notable exceptions in human metabolism to the general rule of organic (nonoxidized) sulfur absorption and inorganic (oxidized) sulfur excretion. The first exception is the excretion of thiol (SH) conjugates of

xenobiotics. The thiol amino acids cysteine and glutathione may combine with xenobiotics (e.g. aromatics) to form thioethers, which are then typically excreted as *N*-acetyl-cysteinyl derivatives in urine or bile. The second exception is the absorption of the beta amino acid taurine, a sulfonic (SO_3^-) acid.

4. Polymorphism in metabolism of S-carboxymethyl-L-cysteine (SCMC) in man

The metabolism of the mucolytic drug S-carboxymethyl-L-cysteine (SCMC) has been studied in man. Its metabolism varies in the population significantly, and this variation is believed to

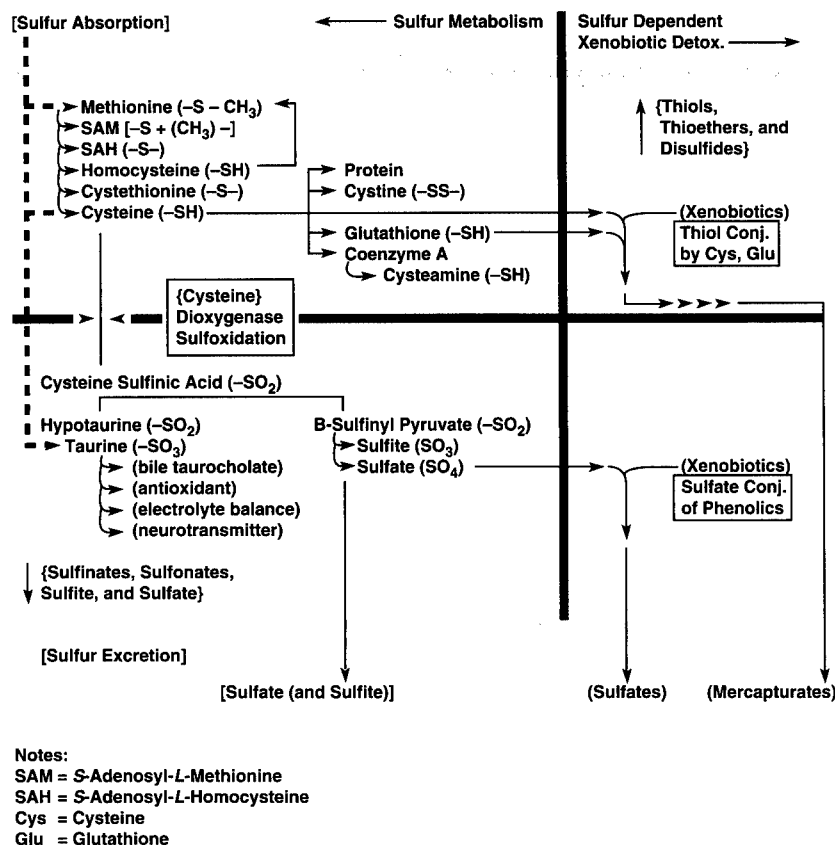


Fig. 1. Major human sulfur metabolism and sulfur dependent xenobiotic detoxification pathways.

reflect a genetic polymorphism (Mitchell and Waring, 1978, 1989a; Turnbull et al., 1978; Waring, 1978, 1980; Waring and Mitchell, 1982; Waring et al., 1982, 1983; Mitchell et al., 1984, 1986; Haley et al., 1985).

Under the standard protocol, a dose of SCMC is given at 0800 h and urine is collected for the following 8 h, after which the metabolites are evaluated by descending paper chromatography. This method is used because it avoids sample degradation, which may occur with some other methods. A number, which has been named the "Sulfoxidation Index" (SI), is calculated by dividing the "nonsulfoxide" SCMC metabolites by the "sulfoxide" SCMC metabolites collected in the 0-8-h urine sample.

Initial data ($n = 200$) indicates that the 'Sulfoxidation Index' is bimodal in the population,

with an additional peak of nonmetabolizers. Initial estimates suggest that there are about 65% good metabolizers ($SI < 6$), 32.5% poor metabolizers ($6 < SI < 80$), and about 2.5% nonmetabolizers ($SI > 80$) in the general population (Mitchell and Waring 1989a; Steventon et al., 1990a).

These researchers believe that this variation in SCMC metabolism implies a polymorphism in the sulfoxidation of the amino acid cysteine to inorganic sulfate by the enzyme cysteine dioxygenase. Impaired sulfoxidation of cysteine is hypothesized to lead to an excess of cysteine and thiol compounds and a deficiency of sulfate in the body, with consequent metabolic effects (Fig. 2). Further, an elevated cysteine:sulfate ratio has been found to correlate with reduced SCMC metabolism in a number of studies.

There has been, however, some controversy about this interpretation. The validity of the test for measuring SCMC metabolism, and its interpretation, have both been questioned. The length of the test, the potential for interfering dietary sulfur compounds, and the possible effects of bacterial metabolism all may be confounding factors (El Fatih et al., 1989; Kupfer and Idle, 1990; Meese et al., 1990; Staffeldt et al., 1990; Waring and Mitchell, 1990; Brockmoller et al., 1991; Hofman et al., 1991; Meese et al., 1991; Gregory et al., 1992; Waring et al., 1992). In addition, admittedly, the extent to which the variation in SCMC metabolism may extend to the sulfoxidation of cysteine and the bulk of sulfur metabolism is not known (Mitchell and Waring, 1989a). However, it is argued that the usual SCMC test protocol adequately reflects the clinically relevant cysteine-to-sulfate ratio.

There has also been some controversy regarding the cysteine-to-sulfate ratio, given the reactivity of cysteine, and the difficulty of storing and measuring it in its free form (Perry et al., 1991a,b; Steventon et al., 1991a). If a sample is not completely deproteinized before freezing, or if an amino acid analyzer is used for analysis (as is commonly done for other amino acids), the reactive thiol cysteine forms stable disulfide dimers such as cystine, so that it is no longer distinguishable from the background of normally occurring dimers.

The most pointed attack on this interpretation, however, is a study suggesting that the "sulfoxide" spot identified as SCMC sulfoxide in SCMC studies using descending paper chromatography is not SCMC sulfoxide, but rather carboxymethylthio-L-cysteine (CMTC), which is thought to be produced not by sulfoxidation, but rather by a transsulfuration process (Gregory et al., 1993). It is additionally argued that, because of the intense color produced by the latter compound in the test system, a small amount of CMTC was mistakenly believed to be the major metabolite. Further investigations seeking to directly evaluate the rate of sulfoxidation of cysteine will need to use a different probe drug if this study is valid.

This is not to say that there is not a polymor-

phism of metabolic importance operant in SCMC metabolism, or that the previously developed SCMC data may not indirectly correlate with an elevated cysteine-to-sulfate ratio of clinical significance. Such an indirect link might hypothetically occur because of correlation between elevated cysteine:sulfate ratios and decreased formation of CTMC for whatever reason. It would seem, rather, that the question of the specific metabolic interpretation of the existing SCMC data is still open for discussion, and that further studies should include measurement of the clinically significant cysteine-to-sulfate ratio.

Overall, however, the evidence for a polymorphism in SCMC metabolism, as reviewed by Mitchell et al. (1992), is fairly impressive. The evidence that the SCMC test protocol specifically probes the cysteine dioxygenase metabolic pathway of liver cytosol, and that this pathway completely reflects both the catabolism of thiols and production of sulfate in the body, is somewhat less so, in part because of the limited extent of current knowledge regarding mammalian sulfur metabolism in general. Liver transplant studies do, however, suggest that SCMC metabolism is primarily controlled by the liver (Olomu et al., 1988). It may be that reduced SCMC metabolism under the standard test protocol should be considered a biomarker rather than a direct probe.

More research in this area would clarify and resolve some of these issues and improve the interpretation of the existing studies. It is notable that research on SCMC metabolism has been limited in the United States to some extent, in part because the sale of the drug SCMC has not been approved (although it is sold as an over-the-counter mucolytic in Europe) — perhaps in part because of its polymorphous metabolism.

5. Sulfation of phenolic xenobiotics in man

Sulfation is a limited-capacity xenobiotic conjugation pathway that is present in many tissues, but, importantly, it acts as a first-pass barrier to the absorption of dietary phenolics in the intestinal mucosa. By contrast, glucuronidation is a high-capacity conjugation pathway in liver, kidney, and the gastrointestinal tract.

The sulfate conjugation of phenolics (aromatic hydroxyl groups) is an important pathway for the detoxification of catecholamine neurotransmitters, steroids, bile acids, and many phenolic and aromatic drugs and xenobiotics (Roth and Rivett, 1982; Levy, 1986; Weinshilboum, 1986; Zou et al., 1990; Rogers et al., 1991). Dopamine, epinephrine, progesterone, and DHEA are some of the endogenous mediators that may be sulfated.

The sulfotransferase enzymes are usually classified for convenience by their substrate, e.g. phenols, monoamines, or steroids. Their action tends to be somewhat nonspecific, and a substrate may be metabolized by more than one enzyme, while most research typically characterizes action with respect to only a single substrate (Roth and Rivett, 1982). Two general phenol sulfotransferase (PST) enzymes exist in all tissues studied, a thermostable enzyme, also known as TS PST or PST-P, which acts primarily on simple phenols, and a thermolabile enzyme, also known as TL PST or PST-M, which acts primarily on catecholic or phenolic monoamines such as dopamine and tyramine (Weinshilboum, 1992). Both forms may be measured in platelets, both forms are polymorphous in the human population, and both may use acetaminophen as a substrate (Emery, 1993). Platelet activity of the two forms do not correlate with each other, but platelet TS PST activity correlates with that in other tissues, though not TL PST (Weinshilboum, 1992). From family studies, TS PST is thought to be 81% heritable with allele frequencies of 0.20 high activity and 0.80 low activity, while TL PST (PST-M) is thought to be 77% heritable with allele frequencies of 0.08 high and 0.92 low (Weinshilboum, 1992).

The sulfation of phenolics is dependent upon a depletable supply of inorganic sulfate, which is believed to be produced predominantly by the sulfoxidation of cysteine. Serum levels of sulfate are maintained by nonlinear renal conservation (Levy, 1986). Inorganic sulfate must be activated to 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the active sulfate donor, by using two molecules of adenosine triphosphate (ATP). As an example of limited capacity of the sulfation

pathway: the bioavailability of acetaminophen is 90% at doses greater than 1 g, but 63% at doses less than 0.5 g because of presystemic metabolism, implying that high therapeutic doses are more available because they saturate first-pass conjugation pathways (Boobis et al., 1992). Raising the dose of acetaminophen from 5 mg/kg to 20 mg/kg reduces the fraction sulfated (Davies et al., 1994a), and an adult dose of acetaminophen greater than 750 mg may saturate the sulfation pathway (Bradley et al., 1991). Thus, it seems likely that impaired sulfation of phenolics most often involves starvation of the sulfotransferase enzymes for activated sulfate substrate, which, in turn, presumably reflects a lack of sulfate due to an imbalance between the rate of sulfoxidation of cysteine and the total metabolic demand for and renal clearance of inorganic sulfate.

First-pass sulfation of phenolic food constituents and bacterial fermentation products (e.g. from protein) within the gastrointestinal mucosa is an important factor in the control of dietary xenobiotics, as glucuronidation is negligible in intestinal mucosa (Ramakrishna et al., 1991). Without first-pass sulfation in the gut, phenolic xenobiotics must be detoxified on the first liver pass; otherwise, they will enter systemic circulation. When sulfation is impaired, conjugation of phenolic xenobiotics with glucuronic acid (a metabolite of glucose) can make up for some but not all of the loss in capacity (Levy, 1986). However, a shift from sulfation towards glucuronidation may prolong conjugate half-life in the body somewhat, because of the enterohepatic recirculation of glucuronides back through the digestive tract (Steventon et al., 1990a).

Human studies have demonstrated functional impairments in sulfation capacity in some individuals. The test procedure typically uses the analgesic acetaminophen as a probe drug, which is detoxified by sulfation and glucuronidation. (Additionally, up to 10% of the dose can be oxidized by the cytochrome P450IIE1 and P450IA2 pathways to form the reactive metabolite *N*-acetyl-benzoquinoneimine, NABQI, which must be detoxified by hepatic glutathione conjugation; acetaminophen overdose can deplete hepatic glutathione levels and result in

Table 4
Populations with reduced metabolism of SCMC, impaired sulfation, and/or an elevated cysteine-to-sulfate ratio

Condition of population group	Reduced sulfoxidation	Reduced sulfation	Elevated Cys:SO ₄ ²⁻
1. Neurological			
Alzheimer's disease (AD) (Steventon et al., 1990a; Heafield et al., 1990)	X	X	X
Parkinson's disease (PD) (Steventon et al., 1989; Heafield et al., 1990; Williams et al., 1991)	X	X	X
Motor neuron disease (MND) (Steventon et al., 1988; Heafield et al., 1990; Pean et al., 1994; see also ALS controversy Perry et al., 1991a; Steventon et al., 1991a)	X	X	X
Autism (Waring and Ngong, 1993; O'Reilly and Waring, 1993)	X	X	X
2. Immunological			
Systemic lupus erythematosus (SLE) (Gordon et al., 1992)	X		X
Primary biliary cirrhosis (PBC) (Olomu et al., 1988; Elias and Waring, 1989; Davies et al., 1994)	X		X
Rheumatoid arthritis (RA) (Emery et al., 1992a,b; Bradley et al., 1991, 1994)	X	X	X
3. Xenobiotic associated			
Chlorpromazine jaundice (Watson et al., 1988)	X		
D-penicillamine toxicity in RA (Emery et al., 1984; Madhok et al., 1990)	X		
Sodium aurothiomalate toxicity in RA (Ayesh et al., 1987; Madhok et al., 1987)	X		
4. Environmental intolerance			
Non-IgE (delayed) food sensitivity (Scadding et al., 1988)	X		
Multiple chemical sensitivities (Monro 1994)	Pending publication		

hepatic injury and liver failure (Boobis et al., 1992). The choice of acetaminophen as a probe drug is a decision that is presumably influenced by its approved over-the-counter sale, widespread availability, presumed safe use in traditional "sensitive subpopulations" such as children and pregnancy, and ready acceptance by ethics review panels.

Pregnancy, which results in elevated levels of estrogens, and estrogen-containing birth control pills both reduce the sulfate:glucuronide ratio of acetaminophen (Davies et al., 1994b). Sulfation is

decreased on the pill, though not significantly, while glucuronidation is increased even more. Postpartum brings a rebound in sulfation while glucuronidation is still high. Platelet PST levels (using phenol as a substrate) are decreased during pregnancy, are increased postpartum, and are increased nonsignificantly on the pill. (The renal clearance rate of acetaminophen in females taking oral contraceptives is 30% higher than those not, in significant part because of increased glucuronidation (Boobis et al., 1992)). Impaired sulfation of estrogens and monohydroxy bile

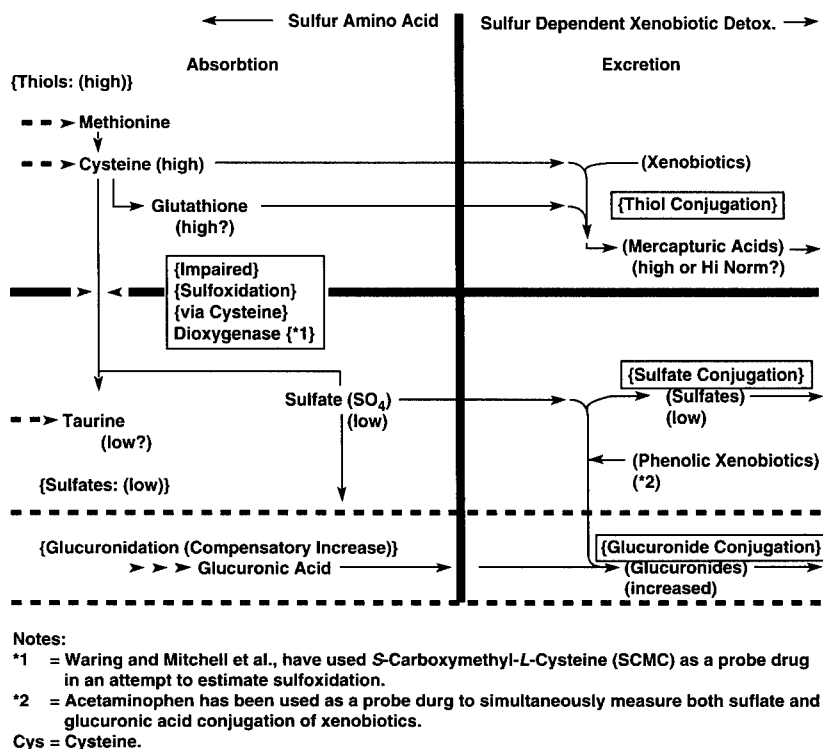


Fig. 2. Potential influence of reduced sulfoxidation of cysteine upon xenobiotic metabolism.

acids by steroid sulfotransferases has been hypothesized to be a factor in the intrahepatic cholestasis of pregnancy (Davies et al., 1994b).

Further studies of individuals with impaired sulfation would seem profitable, in order to further consider factors such as the sulfoxidation of cysteine, renal clearance of sulfate, PST activity, effect of steroid levels, and other relevant factors. Some have suggested, for instance, that an energy metabolism defect may be relevant in ulcerative colitis (Ramakrishna et al., 1991).

6. Studies on SCMC metabolism and sulfation in neurological disease

While a direct link between reduced SCMC metabolism and impaired sulfoxidation of cysteine may not be proven, a close association with an elevated cysteine-to-sulfate ratio is seen in many

studies. Any impairment in the sulfoxidation of cysteine can be expected to alter the balance of xenobiotic metabolism (Fig. 2). It seems likely that thiol conjugation of xenobiotics by cysteine and glutathione would be enhanced, while a lack of available inorganic sulfate would reduce the sulfation of phenolics, though the latter may be compensated for somewhat by a shift in the sulfate:glucuronide ratio towards glucuronides.

Metabolism of SCMC and sulfation of acetaminophen have been studied in Parkinson's disease (PD), Alzheimer's disease (AD), and motor neuron disease (MND) (Table 4). These studies focused on, (1) people who are non-metabolizers of SCMC, defined to be those with a SI > 80, and (2) those with reduced sulfation of acetaminophen, defined as either excreting 5% or less of the dose as the sulfate conjugate, or having a dose sulfate:glucuronide excretion ratio of less than 1.5.

SCMC nonmetabolizers included 35.3% ($n = 68$) of those with PD, compared with 2.5% of the general population and 4.9% of hospital controls. Greater than 5% of the acetaminophen dose was recovered as the sulfate in 29.6% ($n = 27$) of those with PD, compared to 83.9% of the control population ($n = 35$). A sulfate:glucuronide ratio exceeded 1.5 in only 37% ($n = 27$) of those with PD, compared to 70.5% ($n = 50$) of the control population (Steventon et al. 1989, 1990a,b; Williams et al., 1991).

SCMC nonmetabolizers included 62.5% ($n = 16$) of those with AD, while more than 5% of the acetaminophen dose was recovered as the sulfate in only 41.7% ($n = 12$), with a sulfate:glucuronide ratio exceeding 1.5 in only 8.3% ($n = 12$) of this group (Steventon et al., 1990a).

SCMC nonmetabolizers included 46.9% ($n = 49$) of those with MND, while over 5% of the acetaminophen dose was recovered as the sulfate in only 33% ($n = 15$) of this group (Steventon et al., 1988; Pean et al., 1994).

In a separate study, the cysteine:sulfate ratio in PD, AD, and MND patients was found to be increased about a factor of 5 over control ($n = 14-21$) subjects (Heafield et al., 1990; see Perry et al., 1991b regarding order-of-magnitude scale errors in the figures of this paper).

No difference was found in cytochrome P450 hydroxylation of debrisoquine when measured. Red blood cell (RBC) aliphatic thiomethyltransferase (ATMT) activity, a pathway for the detoxification of hydrogen sulfide (which may be produced by intestinal bacteria) and aliphatic thiol compounds (including to some extent cysteine) was measured; this ATMT activity was found high in MND subjects, but low in AD and PD subjects, suggesting that it may protect the nervous system against thiols (Waring et al., 1989; Steventon et al., 1990a; Waring and Emery 1993). Platelet monoamine oxidase B (MAO-B) was found to be increased in PD subjects when measured by using phenylethamine as a substrate, but decreased when dopamine was used as a substrate, which suggests a possible structural polymorphism of the enzyme in PD (Steventon et al., 1990b; Williams et al., 1991).

7. Studies on SCMC metabolism and sulfation in immunological disease, drug reaction, environmental intolerance, and other conditions

Metabolism of SCMC, sulfation of acetaminophen, and/or cysteine:sulfate ratios have been studied in systemic lupus erythematosus (SLE), primary biliary cirrhosis (PBC), and rheumatoid arthritis (RA). SCMC nonmetabolizers included 60% ($n = 35$) of those with SLE compared to 4% ($n = 47$) of controls; and the cysteine:sulfate ratio was 362 ($n = 28$) versus 65 ($n = 30$) for the controls (Gordon et al., 1992). There was no significant difference in ATMT activity. Reduced SCMC metabolism ($SI > 18$) was seen in 84% ($n = 44$) of those with PBC compared to 24% ($n = 66$) of those with liver disease, 22% ($n = 50$) of hospital controls, and 23% ($n = 200$) of normals (Olomu et al., 1988; Elias and Waring, 1989; Scharschmidt and Lake, 1989). Cysteine:sulfate ratios were 0.448 ($n = 34$) in PBC, 0.394 ($n = 26$) in the liver disease group, and 1.650 ($n = 33$) in hospital controls, compared to 0.095 ($n = 40$) in normal controls (Davies et al., 1994a). Reduced SCMC metabolism was seen in RA ("median $SI = 34$ ", $n = 114$), though it did not predict disease activity level (Emery et al., 1992b). An average sulfate:glucuronide ratio of 2.1 ($n = 83$) was seen in RA, compared to 5.6 ($n = 46$) in normal controls and 5.3 ($n = 49$) hospital controls (Bradley et al., 1991). The cysteine:sulfate ratio seen in RA was 446 ($n = 33$) compared to 70 ($n = 33$) for normal controls, and synovial fluid sulfate was also reduced in RA (202 nm) compared to non-RA controls (1041 nm), being roughly twice the plasma levels (Bradley et al., 1994). Reduced SCMC metabolism predicts the development of radiological erosions following the diagnosis of early symmetrical arthritis, with a relative risk of 2.5 (Emery et al., 1992a).

Poor SCMC metabolism ($SI > 6$) was seen in 100% of 12 patients with chlorpromazine jaundice (Watson et al., 1988). Reduced metabolism of SCMC is associated with increased adverse reactions to the thiol drug D-penicillamine in the treatment of RA (3.9 times higher rate for poor metabolizers ($SI > 6$); 7.5 times for $SI > 10$); and

given the prevalence of impaired sulfoxidation in RA, this may explain the increased level of adverse effects seen when this drug was used for RA compared to its use in Wilson's disease or cystinuria (Emery et al., 1984; Waring and Mitchell, 1988; Madhok et al., 1990). Similar results have been found with the thiol drug sodium aurothiomalate (used in "gold" treatments), which is also used in the treatment of RA, where 81% ($n = 37$) of those showing toxicity were poor metabolizers (SI > 6) compared to 32% ($n = 28$) of those not, for a relative risk ratio of 9 (Ayesh et al., 1987; Madhok et al., 1987).

Reduced metabolism of SCMC was found in delayed (non-IgE) food sensitivity (48.6%, $n = 74$; SI > 40 cutoff), a study that included individuals with arthralgia, asthma, migraine, irritable bowel syndrome, perennial rhinitis, urticaria, hay fever, and eczema (Scadding et al., 1988). Reduced first-pass sulfation of acetaminophen in the gut has been found in individuals with both active and quiescent ulcerative colitis, though not Crohn's disease (Ramakrishna et al., 1991). Reduced metabolism of SCMC and impaired sulfation are being studied in multiple chemical sensitivities (MCS) by Monro (1994) in Britain.

Reduced metabolism of SCMC, impaired sulfation, and an elevated cysteine:sulfate ratio are currently being found in autistic children whose symptoms are exacerbated by diet by Waring and co-workers, who are working with parent groups in both Britain and the United States (O'Reilly and Waring, 1993; Waring and Ngong 1993).

Reduced metabolism of SCMC has been found in a pottery worker whose fingerprints produced black specks on clay pottery when it was fired (Harris et al., 1986). No correlation with SCMC metabolism capacity was found in a study of those with odorous urine following asparagus ingestion (Mitchell et al., 1987). Little correlation was found in those with toxic side-effects from the sulfa drug sulphasalazine when used in the treatment of RA, though interestingly, sulphasalazine treatment in RA does elevate plasma thiols, which may have a protective antioxidant effect (Marabani et al., 1987; Pullar et al., 1987).

8. Discussion. Interpretation of research on reduced SCMC metabolism and impaired sulfation: deductions, limitations of research, potential mechanisms of susceptibility, applicability to other populations, and potential therapies

8.1. Preliminary deductions regarding the effect of reduced SCMC metabolism and impaired sulfation

The reduced metabolism of SCMC, impaired sulfation, and elevated cysteine:sulfate ratio seen in many individuals with Parkinson's, Alzheimer's, and motor neuron disease suggest that in a significant subpopulation these conditions may be either produced or exacerbated by metabolic imbalances having neurological effects, whether due to direct toxicity or to impaired metabolism of toxic xenobiotics. Such mechanisms might possibly include the effects of elevated thiols, for example, on neurotransmitters or receptors, or impaired sulfation of neurotoxic xenobiotics. Some have speculated that the finding of elevated ATMT in MND patients raises the possibility that ATMT may protect the central nervous system from damage by thiols (Waring et al., 1989).

Preliminary findings of a similar nature in many individuals with autism suggests that, in extreme cases, these imbalances may affect the metabolism of endogenous neurotransmitters. Sulfation is an important detoxification pathway for monoamines and their metabolites, and human brain neurons contain phenol sulfotransferase enzymes (Roth and Rivett, 1982; Zou, 1990).

The reduced SCMC metabolism and elevated cysteine:sulfate ratio seen in many individuals with systemic lupus erythematosus, primary biliary cirrhosis, and rheumatoid arthritis suggest that in a subpopulation these conditions may be either produced or exacerbated by metabolic imbalances having immunotoxic effects, whether due to direct toxicity or to impaired metabolism of toxic xenobiotics. Such mechanisms might possibly include the effects of elevated thiols, for example, on protein synthesis required for tissue repair, or impaired sulfation of phenolic immunotoxins.

The reduced SCMC metabolism seen in many individuals with chlorpromazine jaundice, as well as toxicity to the thiol drugs D-penicillamine and sodium aurothiomalate used in RA, suggests that these imbalances may reflect an inability of the liver in these individuals to detoxify certain thiol-containing xenobiotics.

Research showing impaired sulfation in ulcerative colitis, ongoing research in showing impaired sulfation in multiple chemical sensitivities, and prior research showing reduced metabolism of SCMC in non-IgE (delayed) food sensitivity suggests that, as a minimum, impaired sulfation of ingested phenolics and phenolic food constituents may be a significant factor in the health problems of individuals complaining of intolerance of foods and environmental chemicals.

8.2. Limitations of research

Several limitations must be noted in the research in this area to date.

(1) The significance of reduced SCMC metabolism is not clear, as the hypothesis that it directly probes cysteine dioxygenase sulfoxidation capacity, and thus is directly associated with an elevated cysteine to sulfate ratio, has been questioned (Gregory et al., 1992, 1993; Waring et al., 1992). A new probe drug may be needed to evaluate sulfoxidation status.

(2) The correlation between disease and reduced SCMC metabolism and impaired sulfation in these conditions is not absolute; typically 30-60% of these groups are nonmetabolizers of SCMC, compared to about 2.5% of controls, and about 60-90% are impaired sulfaters, versus 20-30% of controls.

(3) The condition of reduced SCMC metabolism or impaired sulfation in an individual does not necessarily imply that an individual will develop a debilitating disease. Exposure to a xenobiotic toxicant may be requisite for the development of many of the degenerative diseases discussed.

(4) Little research has been done on the influence of nutrition upon these conditions. It may be that certain nutrient deficiencies can exacerbate these conditions in predisposed individuals, implying that some cases may respond favorably to appropriate nutritional therapy. Current knowledge is

limited in this clinically relevant area.

(5) The possibility exists that some chronic diseases could worsen the metabolic conditions reflected in reduced SCMC metabolism, and thus, with limited research and small sample size, genetic causality of these conditions cannot be considered proven on the basis of existing research with SCMC. Note that cysteine dioxygenase is a very unstable enzyme, requiring a distinct cytoplasmic protein called protein-A to prevent oxidative inactivation.

(6) Research in this area is just beginning, and the list of medical conditions to which this research may be relevant is not complete. This list may be strongly influenced by researcher interest, cohort availability, and the constraints of funding.

8.3. Possible linkage mechanisms between reduced SCMC metabolism and impaired sulfation and chronic disease and adverse environmental response

The links between reduced metabolism of SCMC and impaired sulfation and chronic disease and adverse environmental response have yet to be studied in detail. Possible links include the following:

8.3.1. Impaired detoxification of xenobiotic toxins due to reduced sulfation

Following ingestion of phenolic xenobiotic toxins, reduced sulfation might result in reduced first-pass conjugation in the gut and liver, thus allowing increased systemic absorption, and a reduced rate of conjugation in the liver, allowing increased duration of systemic exposure, together resulting in a substantially greater dose-time exposure product. This might be compounded somewhat by increased enterohepatic recirculation due to a shift from sulfate conjugation to glucuronidation, thus affecting clearance rate and pharmacokinetics. These processes might lead to a significantly increased internal exposure to ingested toxins that are normally detoxified by sulfation, and they may be important with toxins such as neurotoxic pyrethrins, or catechol and indole neurotransmitter analogs (Pall et al., 1987; Steventon et al., 1991b). Additionally, a partial shift from sulfation toward glucuronida-

tion might potentially affect the presumed pharmacokinetics and pharmacodynamics of a wide range of drugs.

8.3.2. Possible effects on endogenous nutrients

Consequent alterations of xenobiotic metabolism in Phase I activation, Phase II conjugation, or Phase III elimination pathways might have an impact on levels of endogenous neurotransmitters, hormones, and nutrients. Reduced sulfation of phenolics could result in altered clearance of mediators such as monoamines and steroids, particularly if there was competition for sulfate substrate between phenolic xenobiotics and these endogenous mediators. Consideration of the effects of depletion or disruption of the sulfate pool upon monoamine metabolism might be relevant to the treatment of conditions such as diet-responsive autism and Feingold-diet-responsive behavior disorders. Depletion of the sulfate pool might affect the sulfation of endogenous biocomponents such as bile acids and joint glycosaminoglycans, and it thus might be relevant to primary biliary cirrhosis and rheumatoid arthritis (Davies et al., 1994a). Increased thiol conjugation of aromatic nutrients, or thiol chelation of minerals, might lower levels of those nutrients because of increased renal clearance.

8.3.3. Formation of thiol-neurotransmitter conjugates

Elevated thiols might increase the formation of free thiol-catechol neurotransmitter conjugates, which may be of neurological importance (Fornstedt et al., 1986, 1990; Rosengren et al., 1985; Ito et al., 1988; Carlsson and Fornstedt, 1991). Thiol conjugation of oxidized catecholamines is a protective process which proceeds roughly three orders of magnitude faster than cyclization of oxidized catecholamines to form aminochromes (Ito et al., 1988). For instance, oxidized dopamine may form 5-S-cysteinyl dopamine endogenously; it is present in the human substantia nigra at about 2.5% of the dopamine level; and preliminary data suggests that it is resistant to monoamine oxidase (MAO) (Fornstedt et al., 1990; Carlsson and Fornstedt, 1991). Similarly, the DOPA conjugate 5-S-cysteinyl DOPA is thought

to be resistant to DOPA decarboxylase. As another example of the neurological potency of some thiols, the small thiol compound cysteamine, the decarboxylated analog of the amino acid cysteine and a constituent of pantothenic acid and coenzyme-A, readily crosses the blood-brain barrier; and in sufficient dose, it may deplete somatostatin and prolactin levels reversibly (possibly because of an indirect effect upon essential disulfide bonds) and produce behavioral changes in rats (likely due to monoamine effects) (Brown et al., 1985; McComb et al., 1985; Millard et al., 1985; Szabo and Reichlin, 1985; Vecsei et al., 1989). Cysteine and cysteamine both inhibit dopamine-B-hydroxylase in vitro (possibly because of the formation of thiol-catechol conjugates). This effect, were it to occur in vivo, would tend to block norepinephrine synthesis; reduced norepinephrine is seen in vivo with cysteamine but not with cysteine itself (Vecsei et al., 1988). Despite the small amount of cysteamine formed endogenously, its potency in vivo, relative to cysteine, should not be ignored. However, normal metabolism of rantidine was formed in MND subjects, suggesting normal degradation of cysteamine by the microsomal flavin monooxygenase system (Pean et al., 1994).

8.3.4. Cysteine as a potential endogenous excitotoxin

Cysteine is considered an excitatory amino acid. Chronically elevated cysteine might act as an endogenous excitotoxin in that portion of the population who cannot metabolize it well (Olney and Ho, 1970; Olney et al., 1972, 1990; Klingman and Choi, 1989; Olney, 1993, 1994a,b; Porter and Roberts, 1993; Blaylock, 1994). This may cause predisposition to neurological disease by some direct mechanism, or it may indirectly cause an increased susceptibility to exogenous excitotoxins.

Specifically, Olney et al. (1990) showed that cysteine acts as an excitotoxin in infant mice, though not in adults, at high doses (e.g. 1 g/kg) causing damage in areas of the brain not protected by the blood-brain barrier, but at lower doses causing slower, but much more widespread damage. This effect is blocked by the drug MK-

801, an NMDA-subtype glutamate receptor antagonist that is active at the phencyclidine (PCP) site, although at higher doses of cysteine there is also involvement of the quisqualate receptor subtype. (MK-801 is a drug often used in neurological research to block the persistent effects of excitotoxicity, e.g. that of anticholinesterase nerve agents). This suggests that cysteine has neurobiological activity via the glutamate receptor.

It is notable that Bell and co-workers have applied the kindling model of excitotoxicity first developed by Goddard as a model of epilepsy, and that of time dependent sensitization (TDS), to allergic sensitization to xenobiotics and the induction of multiple chemical sensitivities (Goddard et al., 1969; Goddard, 1983; Bell et al., 1992, 1993a). In addition, Bell et al. (1993b) have studied cacosmia (adverse response to odors) and olfactory deficits in the elderly, concluding that these may help predict a pattern of memory dysfunction more consistent with dementia (such as that of AD and PD) rather than depression. This research was further developed at a recent meeting on neurobiologic sensitivity (Mitchell and Price, 1994). In short, any neurobiological activity of cysteine via the glutamate receptor could potentially contribute to adverse environmental response to xenobiotics via a neurological mechanism.

Further research may confirm that there exists a human subpopulation with chronically elevated cysteine, possibly due to impaired sulfoxidation, which may act in those individuals as an endogenous excitotoxin predisposing towards neuronal sensitivity, such as by action at the glutamate receptor. Over the course of a lifetime, this increased neuronal sensitivity may result in increased sensitivity to unique stimulus or increased susceptibility to neurological disease. This would have a number of potential implications for health policy, because any such excitotoxicity would likely be synergistic with a number of economically important exogenous excitotoxins in the human environment. Exogenous excitotoxins of concern include anticholinesterase agents and many other neurologically acting insecticides, many agents known

to cause chemical kindling, and some excitatory food constituents. Thus policies relating to chemical defense, insecticide use, neuro- and psychoactive drugs, and food additives would need to consider any such sensitive subpopulation.

8.3.5. Possible alteration of protein structures or enzyme function by abnormally elevated thiols

The formation of cross-links between the SH groups of cysteine amino acids to form disulfide bridges is an important process for maintaining the 3-dimensional structure of many proteins and enzymes. A cysteine thiol group is also the active site of some enzymes. Abnormally elevated thiols may possibly affect protein synthesis or enzyme function through disulfide bonding to the cysteinyl groups at structurally or enzymatically important sites, or by acting upon existing disulfide bridges. Elevated cysteine can interact with immunoglobulins and components of the complement pathway to reduce the clearance of immune complexes, a process which may be important in rheumatoid arthritis (Bradley et al., 1994).

8.3.6. Possible effects of taurine deficiency

To the extent that reduced SCMC metabolism, impaired sulfation, and/or an elevated cysteine:sulfate ratio reflect reduced sulfoxidation of cysteine, a reduction in taurine levels might result. If this were the case, supplementation of taurine might reduce the load on the sulfoxidation pathway. Taurine is a sulfur amino acid used as a constituent of bile (taurocholate), as an inhibitory neurotransmitter, and in maintaining electrolyte balance. Taurine also acts as an antioxidant in phagocytes to quench oxidants produced in immune respiratory burst (Babor and Crowley, 1983). An individual with impaired sulfoxidation might be more susceptible to some forms of allergic exposure and oxidant stress, which would thus compound any sensitivity to ingested phenolics due to impaired sulfation.

8.4. Potential applicability of this research to other populations

(1) There is potential applicability of this re-

search to a better understanding of phenol intolerance. Impaired sulfation of phenolics might result in adverse reactions following the injection or ingestion of phenol-preserved medicinals. Specifically, this suggests a possible mechanism for reports of multiple chemical sensitivities exacerbated by the use of phenol-preserved allergy shots (Rogers, 1986). The routine use of phenol in the preservation of drugs and injectables should be reevaluated in light of this potentially sensitive subpopulation.

(2) Childhood behavior disorders: impaired sulfation of phenolic food constituents may explain part of the success of the Feingold diet, which seeks to control behavior disorders such as hyperactivity and Attention Deficit Disorder (ADD) in children through the avoidance of salicylates, food colorings and preservatives (Feingold, 1974). Salicylate itself is not sulfated, but in rat studies it has been shown to increase renal clearance of sulfate nearly two-fold, primarily by direct inhibition of sulfate transport across the renal basolateral membrane (Darling et al., 1994). Salicylate also inhibits sulfate transport in human placenta, in addition to its effect upon prostaglandin synthesis; and it has been shown to inhibit RBC anion exchange (Shennan and Russell, 1991). Many food colorings and preservatives are aromatics, whose hydroxylated metabolites may be ultimately detoxified by sulfation or glucuronidation.

(3) Impaired sulfation of food constituents may help to explain phenylic intolerance, whose treatment includes allergic neutralization to, and avoidance of, foods containing certain aromatic and cyclic constituents, such as cinnamic acid, vanillylamine, and eugenol (Dadd et al., 1980; Brostoff, 1987).

(4) Impaired sulfation may be significant in tyramine-dependent migraine, given the importance of first-pass sulfation to the inactivation of ingested monoamines (Hannington and Harper, 1968; Littlewood et al., 1982; Davis et al., 1987). Tyramine is a bacterial fermentation product closely related to the catecholamine neurotransmitters, which is often found in cheese, some alcoholic beverages, and many other foods contraindicated during the use of monoamine oxidase (MAO) inhibitor antidepressants be-

cause of the impact of dietary monoamines (e.g. tyramine, phenylethamine, dopamine, and octopamine) upon blood pressure when MAO is low (Preston and Johnson, 1990). Low activity of phenol sulfotransferase, particularly of PST-P, has been found in tyramine-dependent migraine, but curiously, it is PST-M that metabolizes tyramine (Littlewood et al., 1982). It may be that other flavinoids in these foods inhibit PST activity (Waring and Elias, 1993). We suggest that the treatment of tyramine-dependent migraine with high doses of acetaminophen might be contraindicated, because the administration of acetaminophen, which is sulfated and glucuronidated, might deplete the sulfate pool, and thus might impair the sulfation of subsequently ingested tyramine and phenolics.

(5) Some individuals who self-select a vegetarian, low-protein, or other idiosyncratic diet may have experientially found that a balanced low-protein diet is beneficial to their health. Were the sulfoxidation of cysteine to be impaired in an individual, a low protein diet might reduce peak exposures to cysteine and thiols. However, a diet deficient in an essential amino acid may actually increase the need for thiol catabolism by impairing protein synthesis; this has been shown in rats fed a high-gluten diet deficient in lysine (Yamaguchi et al. 1985; Hosokawa et al., 1988). It is interesting that a low-protein diet during daytime has been found useful in the treatment of the "on/off" phenomenon which occurs after several years in those taking L-dopa for Parkinson's disease, although the usual explanation (but incomplete—because it does not explain why the phenomenon develops only later) is that there is competition between L-dopa and large neutral amino acids in both gastrointestinal absorption and in brain penetration (Eriksson et al., 1988; Riley and Lang, 1988).

(6) This research may elucidate the potential for adverse effects of acetaminophen. Acetaminophen is sulfated and glucuronidated, but sulfation is dependent upon an adequate supply of sulfate substrate. The use of acetaminophen at high doses saturates sulfation capacity, and it thus can be expected to impair the first-pass sulfation of phenolics absorbed by the gut. Its overuse may be able to deplete the sulfate pool, and chronic

high-dose treatment might thus impact the amount of sulfate available for other physiological processes. Such treatment may thus be inadvisable for many individuals who have environmental susceptibility due to impaired sulfation. This may be particularly relevant to those with rheumatoid arthritis, a condition in which acetaminophen is often used, and which has been found to have a high percentage of individuals with impaired sulfation and reduced SCMC metabolism, because sulfate is required for maintaining the structure of glycosaminoglycans within joints, and thus a lack of sulfate may impair joint repair (Bradley et al., 1991, 1994). An investigation of impaired sulfation and/or reduced SCMC metabolism as a factor in the induction of acetaminophen-associated kidney and liver injury might be productive.

8.5. Supportive therapies

Given the limited amount of funding for research in this area and the small amount of research done to date, the therapies proposed here must be considered speculative, contingent upon the correct interpretation of the limited data available, and upon the validity of the conceptual model discussed here. For the most part, proposed therapies are low risk (e.g. avoidance of xenobiotics), though their utility should be confirmed by a positive outcome in a clinical trial. Treatment of any chronic disease or medical condition should be done in the care of a qualified physician.

8.5.1. Therapies for impaired sulfation

Individuals excreting sulfite (SO_3^-) in their urine should be treated for sulfite oxidase deficiency. Molybdenum supplements may be useful.

Individuals with homocysteinuria, as demonstrated on an amino acid chromatogram, should be treated for that condition. This reflects elevated plasma levels of the thiol homocysteine due to disordered metabolism of the methionine to cysteine pathway.

Phenolated allergy extracts and other phenolated medicinals should be avoided.

Avoidance of phenolic compounds requiring sulfation (and glucuronidation) may be useful.

These include the drug acetaminophen, which may deplete the sulfate pool.

Salicylate is known to disrupt the sulfate pool by inducing renal excretion of sulfate. The Feingold diet, avoiding salicylates, food colorings, and preservatives, has been found useful by some parents in treating children with autism and behavior disorders. Autism, Intolerance, and Allergy Network-USA (AIA-USA) is working with the Feingold Association of the U.S. (FAUS) in this area (AIA-USA, 1995; FAUS, 1995).

Monoamine-containing foods, such as cheese (tyramine), chocolate (phenylethamine), bananas, and other foods listed as being contraindicated in those taking MAO inhibitor drugs, should be avoided if a physiological effect is noted.

Waring states that some flavinoids, especially those in oranges, result in a temporary reduction in sulfation capacity, and should be avoided (Waring, 1995).

A dietary evaluation to determine if phenolic food constituents common to some food families should be avoided may also be useful (Dadd et al., 1980).

Oral supplementation of a small amount of magnesium sulfate has been reported by some to be useful. The bowel absorption capacity for sulfate is limited, and higher levels may have laxative effect (e.g. as seen with milk of magnesia), or an adverse gastric effect. Some parents of children with diet responsive autism have found that magnesium sulfate baths may alleviate acute symptomatic reactions in those children (AIA-USA, 1995). Many spa waters that have a high sulfate/sulfur ratio have been recommended for therapy for those with rheumatoid arthritis (Waring and Elias, 1993). Some individuals with chronic fatigue syndrome (CFS) have found magnesium sulfate injections to be useful, and it is notable that a significant fraction of individuals with CFS also have symptoms of environmental intolerance.

8.5.2. Therapies for impaired sulfoxidation

To the extent that reduced metabolism of SCMC, impaired sulfation, or an elevated cysteine:sulfate ratio reflects impaired sulfoxidation of cysteine, the following therapies may be relevant.

A low-protein diet, as well as avoidance of supplementation of the amino acids cysteine, cystine, methionine, and other thiol-containing nutrients, may be useful if care is taken to avoid nutrient deficiency. The goal of this is to avoid peak levels of thiol compounds. It has been reported that Parkinsonians tend to avoid green leafy vegetables that contain high levels of organic sulfur compounds. It may be possible that a balanced nutrient supplementation program might in some individuals improve sulfoxidation status, or induce positive compensatory changes. The current state of the art of medicine is limited in this clinically relevant area. Nutrient deficiencies induced by dietary restriction may complicate symptomatic conditions. Avoidance of thiol compounds must not be taken too strictly, especially because a deficiency of essential amino acids can increase the need for thiol catabolism by inhibiting protein synthesis.

To the extent that these conditions may reflect elevated levels of cysteine, and to the extent that cysteine may act as an excitatory amino acid, a trial of avoiding excitatory food constituents, additives, drugs, and toxins may be useful. This might include avoiding foods that are supplemented with the amino acids cysteine, glutamate, and aspartate in free form (e.g. in the flavor-enhancer monosodium glutamate, and in nutrient supplements chelated with aspartate), avoiding stimulants, and avoiding exposure to anticholinesterase insecticides.

Taurine supplementation might support taurine-dependent metabolic processes, thus reducing the load on the sulfoxidation pathway. Note that taurine is a beta amino acid, which shares a common, weak renal conservation pathway with the other beta amino acids, and that excess in any of these compounds will cause renal excretion of all of them (e.g. beta amino aciduria) (Babor and Crowley, 1983). Excess taurine supplementation (more than 2-3 g per day) has a diuretic effect. Dietary intake of the amino acids anserine and carnosine, for example, from chicken and turkey, in individuals with a vitamin B6 deficiency, will cause temporary renal excretion of the amino acid β -alanine, which may induce the renal excretion of taurine and result in

a temporary deficiency state, thus temporarily increasing susceptibility to allergic stress.

9. Conclusions

Science is increasingly able to identify predisposing factors contributing to disease and adverse environmental response as the result of toxic exposure. The study of genetic polymorphisms in xenobiotic metabolism will be an important area of future research.

The finding of reduced metabolism of *S*-carboxymethyl-L-cysteine (SCMC), impaired sulfation of acetaminophen, or an elevated cysteine-to-sulfate ratio in a significant fraction of several groups of individuals with neurological and immunological disease, drug intolerance, and adverse environmental response suggests that such metabolic imbalances may reflect significant predisposing factors toward these and similar conditions.

The finding of impaired sulfation of acetaminophen in ulcerative colitis and in many individuals with multiple chemical sensitivities, and reduced SCMC metabolism in non-IgE-delayed food sensitivity, suggests that this research may be relevant to other groups with sensitivity to phenolics, including phenol intolerance, phenylic allergy, tyramine-dependent migraine, and Feingold diet responsive behavior disorders.

Further studies to more definitively explain the basis for the polymorphism in SCMC metabolism, to probe more directly the cysteine dioxygenase sulfoxidation pathway, and to consider the physiological significance of elevated cysteine and depressed sulfate levels are indicated. Studies of individuals with impaired sulfation, considering the total sulfate balance including sulfoxidation, renal clearance, enzyme polymorphisms, and other factors, would also be of value.

A systematic screening panel for the rapid and inexpensive evaluation of xenobiotic metabolism status should be developed as a basic tool of epidemiology in order to study groups complaining of adverse environmental response. This would improve public health, reduce iatrogenic illness and the costs of treatment of chronic disease, and promote societal understanding of

biochemical individuality and the diversity of the human genome. Advances in genetics, including the Human Genome project, can be expected to improve our understanding of the genetic factors in xenobiotic metabolism in the future.

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SESSION II
MULTIPLE CHEMICAL SENSITIVITY:
CLINICAL, EXPERIMENTAL, AND THEORETICAL
CONSIDERATIONS



ELSEVIER

Toxicology 111 (1996) 69–86

TOXICOLOGY

Chemical sensitivity: symptom, syndrome or mechanism for disease?¹

Claudia S. Miller*

Department of Family Practice, Environmental/Occupational Medicine, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284-7794, USA

Abstract

Several different meanings have been attached to the term “chemical sensitivity” by those who use it. Feeling ill from odors is a *symptom* reported by approximately one-third of the population. The *syndrome* of chemical sensitivity, frequently called “Multiple Chemical Sensitivity” or “MCS” has been the subject of three federally-sponsored workshops; at least five different case definitions for research on MCS have been proposed. In contrast, the hypothesis that chemical sensitivity may be a *mechanism for disease* posits that a broad spectrum of “recognized” chronic illnesses, ranging from asthma and migraine to depression and chronic fatigue, may be the consequence of environmental chemical exposures. According to this theory, a two-step process occurs: (1) an initial salient exposure event(s) (for example, a one-time, intermittent, or continuous exposure to pesticides, solvents, or air contaminants in a sick building) interacts with a susceptible individual, causing loss of tolerance for everyday, low level chemical inhalants (car exhaust, fragrances, cleaning agents), as well as for foods, drugs, alcohol, and caffeine; (2) thereafter, such common, formerly well-tolerated substances trigger symptoms, thus perpetuating illness. “Masking” (acclimatization, apposition, and addiction) may hide these exposure-symptom relationships, thus obfuscating the environmental etiology of the illness. Accumulating clinical observations lend credence to a view of chemical sensitivity as an emerging theory of disease causation and underscore the need for its testing in a rational, scientific manner. While chemical sensitivity may be the consequence of chemical exposure, the term “toxicant-induced loss of tolerance” more fully describes the two-step process under scrutiny.

Keywords: Chemical sensitivity; Theory for disease; Environmental exposure; Environmental etiology; Toxicant-induced loss of tolerance

*Corresponding author, Tel.: 210-567-4577; Fax: 210-567-4579.

¹Presented in part at the Tri-Service Toxicology, US Environmental Protection Agency, and Agency for Toxic Substances and Disease Registry Interagency Conference on Risk Assessment Issues for Sensitive Human Populations, Wright-Patterson Air Force Base, Ohio, April 25–27, 1995.

1. Introduction

The Eskimos have many words in their language for “snow” — there is freshly fallen snow, snow with an icy crust on it that crunches underfoot, fine snow, snow that drifts, snow that clings, soft, deep snow, and so on (Woodbury, 1991).

Snow is an integral part of Eskimo life. In the United States, there are now more than a dozen names for chemical sensitivity: some of these names imply a particular cause (e.g. the petrochemical problem, chemophobia), some describe the effect (e.g. universal allergy, multiple chemical sensitivity), and others insinuate a particular mechanism (e.g. immune dysregulation, odor conditioning) (Ashford and Miller, 1991). "Chemical sensitivity" and "multiple chemical sensitivity" ("MCS") appear favored at present. These terms describe the most distinctive feature of the illness without presuming a particular etiology or mechanism. Notably, some feel that even "MCS" is too much of a legitimizing label and have suggested that "multiple symptom complex" might be less objectionable (Gots et al., 1993).

Some physicians and researchers believe that MCS is a psychogenic phenomenon which most closely resembles depression, somatoform disorder or post-traumatic stress disorder. Others think that MCS is caused by chemical exposures and, further, that chemical sensitivity may underlie a host of "recognized" chronic illnesses, including asthma, migraine headaches, depression, fibromyalgia, and chronic fatigue syndrome. Still others remain agnostic, awaiting more evidence before proffering opinions.

Authors of scientific articles have used the term "chemical sensitivity" in different ways and in different contexts to "mean just what they choose it to mean" (Humpty Dumpty, Lewis Carroll). Words have consequences and a shared understanding of the term "chemical sensitivity" is essential if Jabberwockian *nonscience* (read: nonsense) is to be avoided. The purpose of this paper is to explore the meanings that have been imputed to the term "chemical sensitivity," and, by the close of this paper, convince the reader of the seriousness of the current confusion over its meaning, and how more precise terminology could lead to clearer thinking and greater understanding in this area. Risk assessment, the focus of this conference, requires that we "count the bodies" of those who are afflicted. How we define chemical sensitivity greatly affects this count.

The history and phenomenology of chemical sensitivity, as well as the panoply of hypotheses

that have been advanced to explain it are discussed in detail elsewhere. In the past eight years, two reports sponsored by state agencies (Ashford and Miller, 1989; Bascom, 1989), two books written for occupational health professionals and researchers (Cullen, 1987; Ashford and Miller, 1991), and the proceedings of three federally-sponsored meetings that focused on chemical sensitivity (Association of Occupational and Environmental Clinics, 1992; National Research Council, 1992; Agency for Toxic Substances and Disease Registry, 1994) have been published. A review of these and other recent publications on MCS reveals that the term "chemical sensitivity" is most often used in one of three contexts: (1) to describe a symptom; (2) to serve as a kind of shorthand for an allegedly new medical phenomenon or syndrome; and (3) to characterize what some view as an emerging mechanism for disease. Depending upon which of these contexts is meant, the number and types of persons who might be chemically sensitive varies greatly, and there are enormously different consequences for patients, industry, policymakers and risk assessors. In the next sections, we will explore each of these contexts and the consequences which flow from them.

2. Chemical sensitivity: the symptom

The simplest and perhaps least judgmental application of the term "chemical sensitivity" has been its use to describe the symptom of feeling ill from chemical odors. While the word "cacosmia" has been used in the occupational health literature to describe this symptom, neither this term nor the word "dysosmia" fully convey the observation that MCS patients *feel ill* when they confront certain odors. An alternate term that would convey this might be "pathosmia." Illness from odors has been reported in 60% of a group of solvent-exposed workers (Ryan et al., 1988; Morrow et al., 1990). It also has been reported among agricultural workers following acute organophosphate intoxication (Tabershaw and Cooper, 1966) and among workers in chemical warfare agent production facilities (SIPRI, 1975).

Table 1
Frequency of chemical/odor sensitivity in selected populations

Population	n	Question posed	% Answering affirmatively
EPA office workers (EPA, 1989)	3955	Do you consider yourself especially sensitive to ... (various indoor air contaminants)?	31%
Arizona ^a elderly living in planned retirement community (Bell et al., 1994)	192	Do you consider yourself especially sensitive to certain chemicals?	34%
University of Arizona ^a college students in introductory psychology class (Bell et al., 1995)	809	Do you consider yourself especially sensitive to certain chemicals	28%
Rural North Carolinians (Meggs et al., 1994)	1027	Some people get sick after smelling chemical odors like those of perfume, pesticides, fresh paint, cigarette smoke, new carpet, or car exhaust. Other people don't get sick after smelling odors like these. Do any chemical odors make you sick?	33% (39% of women; 24% of men)

^aA haven for pollen-allergy sufferers in the past, Arizona is thought to have the highest percentage of atopic individuals of any state.

How prevalent is the *symptom* of chemical sensitivity, or pathosmia? To date, no randomized, population-based survey that would answer this question for the nation as a whole has been conducted. However, several smaller surveys suggest that 28–34% or approximately one-third of the population considers itself especially sensitive to certain odors (Table 1). Notably, most of the participants in these surveys who reported that certain odors made them feel ill were neither sick nor disabled. Thus, while approximately one in three Americans reports that certain odors cause illness, the majority of these individuals differ substantially from MCS patients. Whether with sufficient exposure any, some, most, or all of these individuals might develop MCS remains to be determined. Nevertheless, it seems fair to say that the symptom of feeling ill from chemical odors is not identical to nor specific for MCS.

Thus chemical sensitivity—the symptom—lacks specificity for the condition under scrutiny. It may also lack sensitivity. That is, individuals who may be chemically sensitive may not be aware that they are chemically sensitive (for example, migraineurs, chronic fatigue sufferers). “Masking” (to be discussed in a later section) may interfere with this awareness. Thus, the

symptom of chemical sensitivity or pathosmia, while prevalent in the general population, is not very specific, and, indeed, also may be insensitive as an indicator of illness associated with low level chemical exposures. Notwithstanding, the term “pathosmia” has value for descriptive clinical and research purposes. Its adoption for describing the symptom of feeling ill from chemical odors may be preferable to the term “chemical sensitivity” which has acquired other meanings, as shall be discussed.

3. Chemical sensitivity: the syndrome

A “syndrome” is “a *group* of symptoms or signs typical of a disease” (Webster’s, 1986). Many have objected to labelling chemical sensitivity a syndrome because the patients report such diverse symptoms. Indeed, while individual patients report that their symptoms occur reproducibly following exposure to specific substances, unlike other recognized syndromes there is no archetypical constellation of symptoms that constitutes MCS. This fact has thwarted development of a case definition for the illness. While a precise MCS case definition would be desirable for research purposes, restricting thinking about

chemical sensitivity to any immutable set of symptoms or number of organ systems could prematurely constrict the field of view. For example, some proposed MCS case definitions exclude asthma or depression on the grounds that these are “diagnosable” conditions. Yet asthma or depression might themselves be the consequence of low level chemical exposure. In contrast, *exposure (or event) driven studies*, for example studies of office workers in a sick building or residents of a community exposed to a toxic spill, could yield a fuller understanding of the range and nature of illnesses that ensue (National Research Council, 1992). More specifically, it is conceivable that exposure to emissions from new carpeting in a poorly ventilated office building could cause or exacerbate a panoply of conditions, with the occupants’ personal (genetic, nutritional, etc.) vulnerabilities determining which specific symptoms manifest in any individual case. Indeed, some researchers have suggested that RADS (Reactive Airways Dysfunction Syndrome), an asthma-like condition that develops after an acute chemical exposure and leaves its sufferers sensitive to chemically-diverse inhalants, may be a pulmonary manifestation of MCS (Meggs and Cleveland, 1993). Still others hypothesize that certain cases of depression and somatoform disorder may be caused or exacerbated by chemical exposures and thus could share the same biochemical underpinnings as MCS (Rosenthal and Cameron, 1991; Bell, 1994). Then there are odor-triggered migraines and seizures. Might these be induced by the same mechanisms that underlie MCS? Limiting the search for chemical sensitivity to any predetermined set of symptoms or clinical criteria makes little sense when we are in such an early observational stage in this illness.

At least five case definitions for research on chemical sensitivity have been published (summarized in Miller, 1994), yet consensus on a single definition has not emerged, again largely because of the heterogeneity of symptoms patients report. Miller and Mitzel (1995) compared two groups of MCS patients—37 who attributed onset of their illness to a well-defined exposure to a cholinesterase-inhibiting pesticide and 75 who

attributed onset of their condition to remodeling of a building. Despite differences in *individual* symptom patterns and different initial exposures, the two *groups* exhibited strikingly similar frequencies and ordering of symptoms. The authors interpreted these findings as suggesting a shared mechanism or final common pathway leading to the same disorder in both groups.

Some view the fact that no symptom-based case definition for MCS has achieved broad acceptance as evidence that the condition does not exist. On the other hand, this lack of consensus could be a serendipitous clue that chemical sensitivity is not a *single* syndrome, but a *collection* of syndromes that share the same general mechanism, much as cholera, influenza and Rocky Mountain Spotted Fever share the same general mechanism, in this case, infectious transmission.

4. Chemical sensitivity: a mechanism for disease?

The Civil War marked the last major armed conflict fought in the world without knowledge of microbes or infectious disease (Sartin, 1993). A few years later, Pasteur, Lister, Koch and others made their discoveries which led to the germ theory of disease. Two-thirds of the approximately 660 000 deaths among soldiers during the Civil War were caused by infections, most frequently wound infections and epidemics. Diagnoses were based on clinical signs. Fever cases were divided into three categories: remittent, intermittent, and relapsing. Cases assigned to those categories likely included typhus, typhoid, malaria, abscesses, tuberculosis, leptospirosis, borrelia, pneumonia, influenza, infectious diarrheas and others. Brucellosis, tularemia, leptospirosis and Q fever likely were present too, but put in other categories such as “miasmatic diseases, unclassified.” Civil War surgeons commonly attributed diseases to toxic miasma or “effluvia” from the wet swamplands of the South where many battles were fought or to inadequate ventilation in the tents (Sartin, 1993).

It is possible that we may be at the Civil War stage in our understanding of chemical sensitivity. Chemical sensitivity could be an emerging mechanism or theory of disease, one that en-

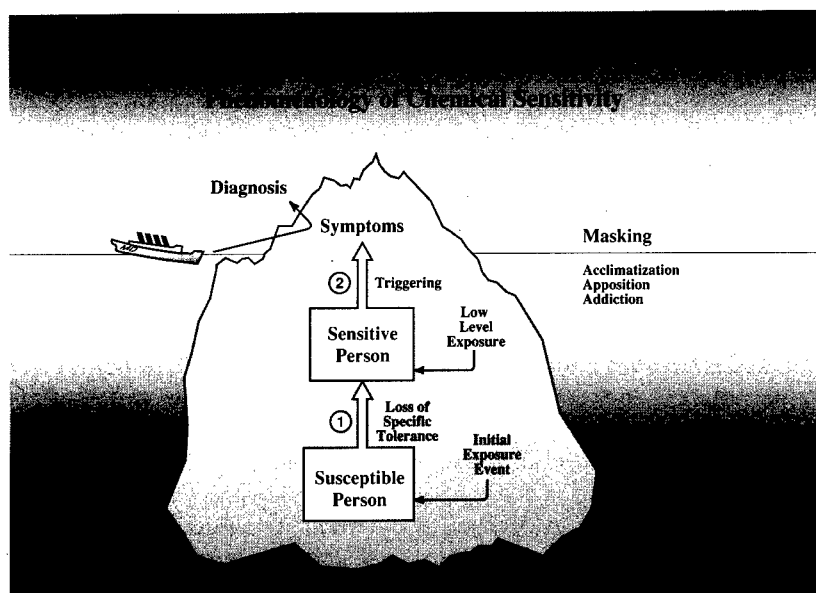


Fig. 1. Phenomenology of chemical sensitivity. Chemical sensitivity appears to develop in two stages: (1) loss of specific tolerance following acute or chronic exposure to various environmental agents, such as pesticides, solvents or contaminated air in a sick building, and (2) subsequent triggering of symptoms by extremely small quantities of previously tolerated chemicals, drugs, foods, and food/drug combinations (e.g. traffic exhaust, fragrances, caffeine, alcohol). Physicians formulate a diagnosis based upon symptoms reported to them by their patients. Because of masking, both physicians and patients may fail to "see" that everyday, low-level exposures may be triggering symptoms. Even when such triggers are recognized, an initial exposure event which may have initiated loss of specific tolerance may go unnoticed or may not be linked to the patient's illness.

compasses a wide range of chemical agents acting on different organ systems via different specific mechanisms, just as the category "infectious diseases" subsumes a wide range of vectors affecting different organ systems via different specific mechanisms resulting in heterogeneous symptoms. Is there any evidence to support such a notion?

If chemical sensitivity were a candidate for a theory of causation for disease, at the very least it would need to embrace MCS, the most prototypical of the chemical sensitivity "diseases," and would need to account for the clinical observations associated with MCS. These observations are discussed in detail elsewhere (Ashford and Miller, 1989, 1991; Miller, 1994) and summarized in Fig. 1. Briefly, chemical sensitivity appears to entail two steps: (1) induction, and (2) triggering. The inducing or initiating exposure may involve any of a wide variety of substances, including

pesticides, solvents, indoor air contaminants, drugs, etc. It may be acute as in a chemical spill, intermittent as in many industrial exposures, or chronic as in a sick building. *Loss of tolerance* appears to occur as a consequence of this initial exposure. Subsequently, extremely low levels of chemicals, levels that do not bother most people and were never a problem for that individual before, trigger symptoms. In addition, alcoholic beverages, caffeine, nicotine, various foods and drugs may trigger symptoms. On the surface, "loss of tolerance" may sound like a vague and ill-defined concept, the product of arm-chair theorizing. Clinically, it is neither vague nor ill-defined. What MCS patients describe is a loss of tolerance for *specific* inhalants, drugs, foods, etc. This loss of tolerance is reported to spread to an increasing number of substances as the problem progresses. When MCS patients are re-exposed to these specific substances, they say they experi-

ence a discrete constellation of symptoms. While the symptoms that are triggered by a given agent are highly individual, they are reportedly reproducible for a particular patient following a particular exposure, for example, headache with diesel exhaust, mental confusion with a certain fragrance, nausea with cashews, and so on. Notably, many MCS patients and Gulf veterans now forego chocolate, favorite foods like pizza, or alcoholic drinks because they say these make them so sick (Miller, 1994). Most of us would not give up these indulgences without good cause—there seems little secondary gain to be garnered from such abstinence, although some psychologists and psychiatrists, no doubt, would argue otherwise.

Thus, loss of specific tolerance is a focused, well-defined construct, one no more fuzzy than the General Adaptation Syndrome and its associated concepts of stress and stressors were when Hans Selye first introduced them.

Chemical sensitivity has been accused of contradicting the fundamental principles of toxicol-

ogy (Waddell, 1993). The same might have been said for allergy (or even cancer) when it was first discovered, and is even true now. Allergy's rules differ greatly from those of toxicology, and, if chemical sensitivity is real insofar as being a disease mechanism, it seems to follow still another set of rules. Chemical sensitivity appears to have major overlaps with toxicity, allergy and addiction (Table 2), yet it does not adhere to all of the rules for any one of these.

Like toxicity, chemical sensitivity appears to involve adverse responses to environmental chemical contaminants, yet, mechanistically, chemical sensitivity more closely approximates allergy in that both involve a two-step (induction, triggering) process and subsequent "hypersensitivity." As with addiction, patients with chemical sensitivity report stimulatory and withdrawal symptoms. Unlike addiction, these responses occur not only to drugs, but also apparently to combustion products, fragrances, solvents and even foods. However, instead of manifesting addicted behaviors (*L. ad* "toward" + *dicare* "pro-

Table 2
Features of chemical sensitivity that overlap addiction, allergy, and toxicity

Feature	Chemical sensitivity ^a	Addiction ^a	Allergy ^a	Toxicity ^a
Chemical/drug intolerances	+	+	+	+
Ambient air incitants	+		+	+
Food intolerances	+		+	
Alcohol intolerance	+	+		
Caffeine intolerance	+	+		
Withdrawal symptoms	+	+		
Craving, bingeing	+	+		
Sensitization	+		+	
Induction by chemicals	+		+	+
Induction by biologicals			+	
Multi-system symptoms	+	+	+	+
Frequent CNS symptomatology	+	+		+
Well-defined mechanism(s)			+	+
Genetic susceptibility	+	+	+	+
Dose/response relationship	+ ^b		+ ^b	+

^aCategories are not "pure" and may overlap in a given host, e.g. haptentation of a chemical toxin may initiate an immunologic response; brain and liver toxicity may accompany alcohol addiction.

^bDose-response does occur for allergens: with the first, sensitizing exposure for a susceptible individual, there is a dose-response relationship; with subsequent exposures, the sensitized person also responds in proportion to dose, but at a much lower dose level (Waddell, 1993). The same kind of dose-response relationship may pertain for chemical sensitivity, but has not been tested. However, individuals with MCS report increasingly severe symptoms the longer they remain in an exposure situation, an observation which hints at a dose-response interaction.

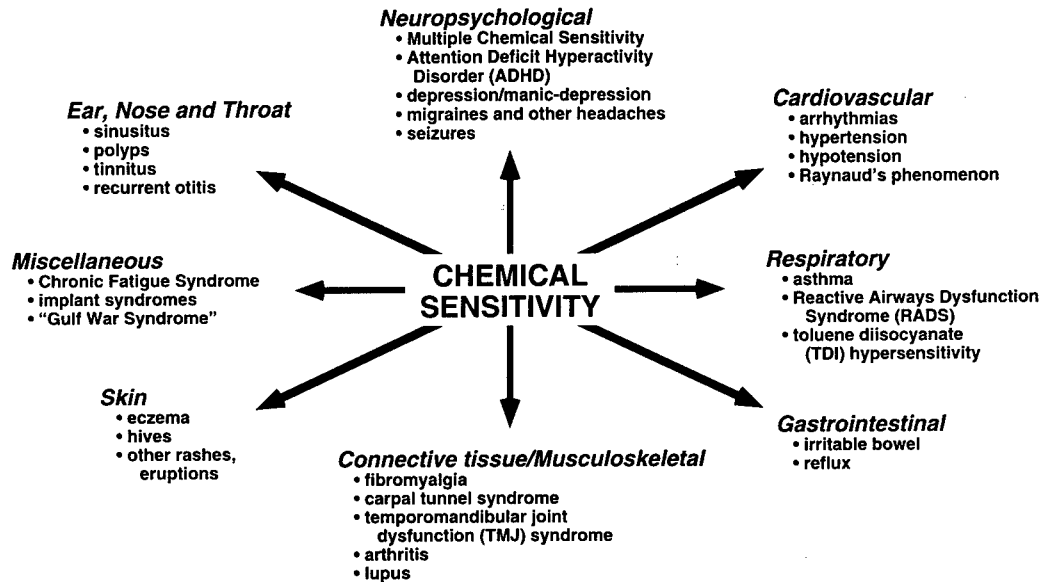


Fig. 2. Conditions that have been attributed to chemical sensitivity. (Of course, illnesses like depression, migraine, arthritis, hives, etc. may have many different etiologies. At most, chemical sensitivity might explain only a subset of these conditions. The question is, for each illness, how large is that subset and how might physicians distinguish those cases from similar cases with different etiologies).

claim”), patients act as though they are *ab*-dicted (L. *ab* “away from” + *dicare* “proclaim”) and assiduously *avoid* the very substances addicted individuals frequently favor, for example, alcohol, drugs, nicotine, and caffeine.

A major criticism of chemical sensitivity is that it has been claimed as a basis for “literally any physical or mental illness” (Waddell, 1993). Indeed, the range of illnesses that have been attributed to chemical sensitivity is enormous (Fig. 2) and involves any and all organ systems. Notably, and perhaps analogously, infectious diseases and immunological conditions also can affect virtually any organ system, so this criticism does not in any way negate the possibility that chemical sensitivity exists. In fact, close inspection reveals intriguing parallels between chemical sensitivity and the germ and immune theories of disease (Table 3). Each of these three theories embraces a wide range of specific agents or exposures. Included under each of these three categories are heterogeneous specific mechanisms (for example, the mechanisms of viruses vs. bac-

teria vs. rickettsia, or immediate vs. delayed-type hypersensitivity). Nonetheless, these diverse mechanisms still fall within the same general category, that is, infectious diseases or immunological diseases. Each of the three categories contains specific named syndromes or diseases. And each category contains processes that can affect literally any organ system. The specific manifestations of a given disease process in each of these categories depend on the nature of the original incitant and the vulnerabilities of the host, whether the category is infectious diseases, immunological diseases or chemical sensitivity. *Between* categories, symptoms are quite similar—malaise, rashes, headaches, shortness of breath, and diarrhea are common, non-specific manifestations for many illnesses. There is nothing pathognomonic about such symptoms. They can accompany an infectious process just as readily as an immunological or toxic one. It is the specific syndromes or named diseases within categories that have recognizable constellations of symptoms which facilitate diagnosis. Thus, rather

Table 3
Comparison of key features of three theories of disease: the germ theory, immune theory and chemical sensitivity theory

Disease theory	Explanation for disease	Symptoms	Particular diseases/syndromes	Agents	Exposure routes	Organ systems affected
Germ (Infectious)	Biological agent or "germ" multiplies in host (infection); germs can be transmitted to other organisms	Fatigue, malaise, fever, rash, headache, nausea, vomiting, diarrhea, shortness of breath, shock	Malaria, influenza, chicken pox, cholera, Lyme disease, AIDS, pneumonia	Bacteria, viruses, parasites, fungi, rickettsia	Inhalation, ingestion, injection, skin/mucous membrane contact	Any organ system or several at once
Immune	Host responds to foreign substance (antigen) by producing antibodies which "remember" the antigen and help ward it off in the future	Malaise, rash, pruritus, rhinorrhea, wheezing, shortness of breath, diarrhea, fever, laryngeal edema, shock	Allergic asthma, hives, poison ivy, penicillin allergy, peanut allergy	Pollen, mold, house dust mite, animal dander, foods, biologicals (drugs)	Inhalation, ingestion, injection, skin/mucous membrane contact	Any organ system or several at once
Chemical Sensitivity	Environmental exposure causes loss of specific tolerance in host, who subsequently develops symptoms when exposed to low levels of chemically diverse substances	Fatigue, malaise, memory difficulties, depression, irritability, headaches, digestive problems, shortness of breath, odor intolerance	Multiple chemical sensitivity, "Gulf War Syndrome" (?), chronic fatigue, syndrome (?), post-implant syndromes (?), reactive airways dysfunction syndrome (RADS)	Solvents, pesticides, combustion products, drugs, implants	Inhalation, ingestion, injection, skin/mucous membrane contact	Any organ system or several at once

than being a syndrome *per se*, chemical sensitivity has many of the earmarks of an emerging category of disease. Such an unproven, yet-to-be-tested general mechanism for a collection of illnesses is a *theory of disease*.

Recalling the critics view that chemical sensitivity is not a syndrome because no definable set of symptoms is associated with it, the critics make an important point: chemical sensitivity may no more be a syndrome than infectious diseases and immunological diseases are syndromes. Nor is there any conceivable case definition that would cover all infectious diseases or all immunological diseases. Case definitions simply have limited utility when we are discussing categories of disease.

Could chemical sensitivity be an emerging new theory of disease? In 1868, while commenting on the usefulness of the germ theory of disease, Max Boehr provided a remarkably insightful and concise synopsis of the criteria for choosing among alternative theories (Carter, 1985). He noted that "the theory of infection has the characteristic of all good pathological and physiological theories; it provides a unified, clear, and entirely intelligible meaning for a whole series of anatomical and clinical facts and for the relevant experiences and discoveries of reliable observers during epidemics." Likewise, chemical sensitivity is a theory of disease that could unite disparate clinical observations and that may enable practitioners to predict patient responses under conditions of

Table 4
Frequency of selected symptoms reported as severe^a by Gulf veterans, MCS patients and controls

Symptom	Gulf veterans (n = 59)	MCS pesticide-exposed (n = 37)	MCS remodeling-exposed (n = 75)	Controls ^b (n = 112)
Fatigue	78%	68%	52%	3%
Depression	29	49	33	6
Headaches	53	38	31	5
Shortness of breath	38	43	31	2
Asthma or wheezing	12	27	15	0

^aParticipants rated their symptoms on a 0-3 scale: 0, not at all a problem; 1, mild; 2, moderate; 3, severe. Frequencies listed in this table reflect ratings of severe (3) only.

^bAge, sex and education-matched to the two MCS groups (37 + 75 = 112).

exposure and avoidance. While clinical observations on MCS accumulated to date do not constitute proof of the condition's existence, they are hypothesis-generating and provide the foundation for what could be an emerging theory of disease, a theory worthy of careful scientific testing.

Some have proposed that the same mechanisms that are operative in chemical sensitivity might underlie conditions like fatigue, depression, headaches and asthma. If one compares the frequency of these particular conditions among groups of ill Gulf veterans and MCS patients to the frequency of these same conditions among controls (matched for age, sex and education to the MCS patients), 5 to 15 times as many Gulf veterans and MCS patients report *severe* fatigue, depression, headaches, shortness of breath, asthma or wheezing as controls (Table 4). Thus, Gulf veterans and MCS patients who attribute their illness to an "exposure event" represent an enriched sample of the population for these conditions. This finding suggests that at least a subset of persons suffering from headaches, chronic fatigue, asthma or depression might be chemically sensitive. It is noteworthy that persons with these conditions often share the same exposures and environments that MCS patients say caused their illness.

Anomalies often pave the way for discovery. The anomalous observation that individuals who survived a particular infection rarely contracted that infection again led to the immunologic con-

cept of disease. The anomaly of MCS could likewise expand our thinking about disease causation. Thomas Kuhn (1970) observed that theories are "generally preceded by a period of pronounced professional insecurity. As one might expect, that insecurity is generated by the persistent failure of the puzzles of normal science to come out as they should. Failure of existing rules is the prelude to a search for new ones." Perhaps, we are in what Kuhn characterizes as a pre-paradigm period, a time "regularly marked by frequent and deep debates over legitimate methods, problems, and standards of solution, though these serve rather to define schools than to produce agreement."

5. Masking

If individuals with migraines, chronic fatigue, asthma or depression were suffering from chemical sensitivity, why wouldn't more of them report specific chemical intolerances just like MCS patients do? In fact, many MCS patients say that when they first became ill, they had no idea chemical exposures had anything to do with their symptoms. They say it was not until they avoided (accidentally or intentionally) a sufficient number of their problem incitants simultaneously that they noticed feeling better. Then, when they re-encountered something to which they were sensitive, their symptoms recurred. Thus, MCS patients claim that only through the tandem process of avoidance and re-exposure were they able to

learn which exposures triggered their symptoms. Had they not systematically avoided a wide range of exposures first, or "unmasked" themselves, accidentally or intentionally, most say they would not have figured out what was making them sick. This lay term, "masking," sounds deceptively simple. In fact, what patients mean by masking is technically quite complex. Understanding masking may be fundamental to understanding chemical sensitivity, its potential role in chronic illness and how such a theory of disease might be tested scientifically. When MCS patients talk about "unmasking," they are describing a complex phenomenon that is poorly understood but experientially recognizable to most of us. There are at least three components involved in masking: acclimatization, apposition and addiction (Fig. 3). "Unmasking," that is, avoiding multiple potential incitants simultaneously, overcomes each of these three components of masking.

The most rigorous approach to unmasking, an approach developed in the 1950s by allergist Theron Randolph, involves placing an individual in an environmentally-controlled hospital unit (not an exposure chamber) and restricting food and drug intake for several days to a week. This approach (described in Ashford and Miller, 1991), eliminates any *acclimatization* that may have developed in a sensitive individual as a consequence of chronic or repeated exposure to an incitant, for example, indoor air contaminants in a sick building. It simultaneously allows the avoidance of inhalation, ingestion and dermal contact for most chemicals, foods, and drugs, thus preventing overlapping symptoms that might occur in a sensitive individual experiencing multiple, overlapping exposures (*apposition*). Finally, this approach interrupts any *addiction* due to repeated exposure to caffeine, alcohol, nicotine or other addictive substances. Fig. 3 depicts each of the components of masking. In effect, masking and unmasking pertain to the *state* of the patient—the underlying illness remains constant. Depending on background environmental conditions, chemical sensitivity seems to occur in masked and unmasked varieties which outwardly appear different but are intrinsically the same.

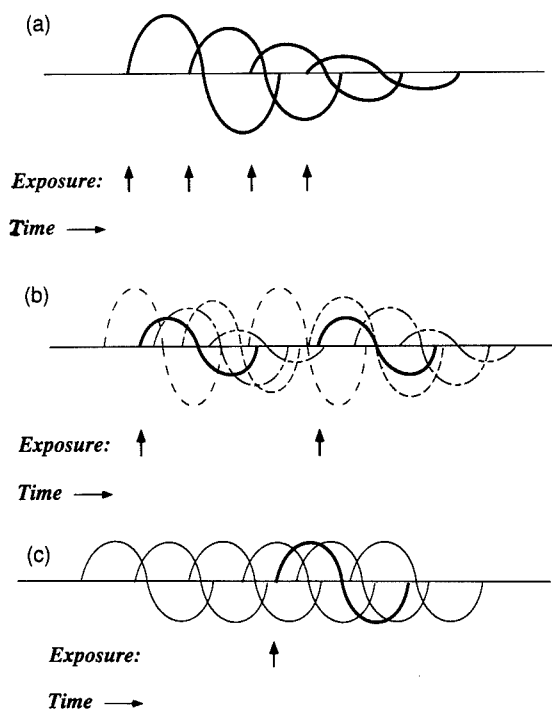


Fig. 3. Components of masking. Each biphasic curve is a graphical representation of symptoms occurring in a sensitive individual with onset and offset of a particular exposure. For solvents, caffeine, alcohol, nicotine, etc., the portion above the line represents stimulatory symptoms and the portion below withdrawal symptoms. Amplitude is proportional to symptom severity. For a person who is not particularly sensitive to the substance, the curves would be flat or nearly flat. Solid curves represent the effect of interest, that is, a response to a particular chemical or food one is trying to observe during a challenge with that substance. (A) Acclimatization: symptom severity decreases with repeated, closely-timed exposures to the same substance. Although seemingly paradoxical, sensitivity may increase during acclimatization. In real life, time intervals between exposure may vary greatly, so that a person may be acclimatized to varying degrees at different times, and thus, from day to day, experience varying intensity of symptoms. However, the most robust effect of a challenge would be expected in the sensitive individual who is not acclimatized. (B) Apposition: if an individual is sensitive to many different substances, then the effects of everyday exposures to chemicals, foods or drugs may overlap in time. This apposition of effects might yield an individual who feels bad most of the time, but the effect of any single exposure is not apparent either to the individual or his physician. Apposition would tend to mask the effect of interest in much the same way that background noise masks a sound of interest. (C) Addiction: a sensitive person who is addicted to caffeine, alcohol, nicotine, or other substances may deliberately take the substance at frequent, carefully-spaced intervals to avoid unpleasant withdrawal symptoms. Such exposures may also mask an effect of interest.

There are no scientific equivalents for the terms “masking” or “unmasking.” “Adaptation” and “de-adaptation” (terms previously used by this author) have other connotations that seem to confuse rather than clarify understanding of this concept; inevitably their use leads to a discussion as to whether adaptation or pseudoadaptation is occurring (for example, in cigarette smokers who develop apparent tolerance for the irritative properties of tobacco smoke, but may experience long-term deleterious consequences from tobacco use). Understanding masking may be crucial to understanding chemical sensitivity. As long as patients with depression, migraines, fatigue, etc. are masked, it may be impossible to tell whether there is any relationship between their symptoms and low level environmental exposures. They must first be unmasked. Testing such patients in a masked state would be like trying to find out whether headaches in a coffee drinker with a 10–15 cup per day habit were due to caffeine by giving him a cup of coffee and asking how it made him feel. It is intuitively obvious that the individual would need to stop using all or most caffeine (unmask him for caffeine) before a meaningful test of caffeine sensitivity could be performed. Falsely negative challenges are likely to result from failure to unmask. This experimental requirement of unmasking brings to mind the enormous difficulty researchers encountered during the late nineteenth century in trying to isolate the organism responsible for tuberculosis. Many researchers collected fluids from TB patients but were unsuccessful in culturing any organism. At the time, these researchers were unaware that the organism was extremely fastidious and would only grow out after weeks on a specialized culture medium. Some who failed to grow the organism concluded that TB was not an infectious disease at all. Correspondingly, our ability to “see” chemical sensitivities may rest upon optimizing experimental conditions, i.e. using the appropriate culture medium, in this case an environmental unit for unmasking and studying this phenomenon. Absent such an approach, erroneous conclusions about the existence of MCS may be reached as a consequence of inadequately

designed experiments resulting in falsely negative data.

6. Testing the theory of chemical sensitivity: the need for postulates

Following the introduction of the germ theory of disease in the late 1800s, many overly enthusiastic researchers employing careless bacteriological technique claimed to have discovered the causative agents for diseases such as TB or Yellow Fever. So frequent were these pronouncements and subsequent retractions that in 1884 the President of the New York Academy of Medicine, Abraham Jacobi, “lamented the ‘bacteriomania’ that had swept the medical profession” (Warner, 1985). In order to preclude further such pseudodiscoveries, Koch, who identified the organisms that cause tuberculosis, anthrax and cholera, suggested adoption of a set of rules for etiological verification, now known as Koch’s postulates:

- (1) The microbe must be present in every case of the disease.
- (2) It must be isolatable in pure culture.
- (3) Inoculating a healthy animal with the culture must reproduce the disease.
- (4) The microbe must be able to be recovered from the inoculated animal and grown again.

In comparable fashion, research on chemical sensitivity today is in need of a set of scientific principles or postulates that will ensure that causal determinations in this area are scientifically based. Fig. 4 illustrates use of an environmental medical unit as a means of testing responses to low level environmental exposures, and depicts four postulates which, if met, would establish proof of a cause-and-effect relationship between symptoms and a particular exposure or incitant, if such a relationship exists. These postulates are:

- (1) When the sensitive individual simultaneously avoids all chemical, food, and drug incitants, remission of symptoms occurs.
- (2) Symptoms occur with reintroduction of an incitant.
- (3) Symptoms resolve when the incitant is again avoided.

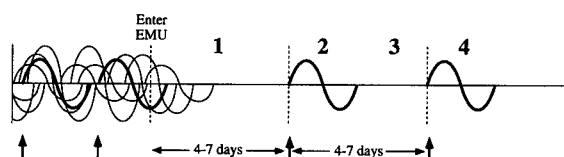


Fig. 4. Graphical representation of chemical sensitivity postulates. In the left-most portion of the figure, before entering an environmental medical unit (EMU), a chemically sensitive individual is experiencing symptoms in response to multiple exposures (chemicals, foods, drugs). Effects overlap in time. The effect of any particular exposure does not stand out from those of other exposures and the person's symptoms appear to wax and wane unpredictably over time. Postulate 1: when all chemical, food and drug incitants are concurrently avoided, remission of symptoms occurs. Postulate 2: symptoms occur with reintroduction of an incitant. Postulate 3: symptoms resolve when the incitant is again avoided. Postulate 4: re-exposure to the same incitant, within an appropriate window of time (estimated to be about 4-7 days), produces the same symptoms. For research purposes, challenges should be conducted in a double-blind, placebo-controlled manner.

(4) With re-exposure to the same incitant, the same symptoms recur, provided that the challenge is conducted within an appropriate window of time (ideally 4-7 days after the last exposure to the incitant).

Note that for research purposes, challenges of this type should be performed in a double-blind, placebo-controlled manner (Ashford and Miller, 1991). This approach could be extended to patients with MCS, chronic fatigue, migraines, seizures, depression, asthma, and other conditions to determine whether or not a particular person's illness is a *forme masquée* of chemical sensitivity.

What evidence is there that unmasking patients in an environmental unit and adhering to a 4-7 day window of time between challenges are important for this process? Only the observations of thousands of credible patients and dozens of physicians who have attempted this maneuver. Physicians and patients who have followed this procedure report impressively robust symptoms following food and chemical challenges. Given that clinical observations are the wellspring of most new medical ideas, we must guard against dismissing these patients' reports without trying

first to recreate the circumstances in which they say their responses are most clearly elicited. Some researchers may argue that an environmental unit and unmasking should not be necessary if patients are experiencing symptoms with daily exposure. It is true that an effect may be seen with exposure challenges outside of a unit. However, certain problems can be anticipated. First, patients' responses will not be as robust if unmasking and appropriate timing between challenges are not considered. But, more concerning, patients' responses may not be reproducible from one day to the next. For example, if patients must drive through heavy traffic to get to an exposure chamber in order to undergo a challenge, their responses to the challenge may be blunted or perhaps disappear entirely. Conversely, if a placebo is administered, sensitive individuals might still be experiencing delayed effects of their exposure to car exhaust while in transit to the laboratory, and falsely positive responses could occur.

7. Chemical sensitivity: the need for new terminology

Applying the moniker "chemical sensitivity" to the theory of disease described above fails to convey adequately either the potentially disabling nature of the condition or its induction by an earlier exposure event. The two-step process that has been described for chemical sensitivity begins with a chemical exposure which appears to be "toxic," that is, it causes adverse effects in at least a subset of individuals (i.e., when they subsequently are exposed to different substances). These individuals report life- and career-disrupting disability following an identifiable initial exposure. While Paracelsus aptly stated that dose makes the poison, in truth, "dose + host" makes the poison (for example, pack-years of tobacco smoked + the α -1-antitrypsin-deficient individual). Admittedly, the nature of the initial "toxicity" in chemical sensitivity departs radically from that of classical toxicity (Table 2). Clinically, this "toxicity" more closely resembles allergic sensitization, although, here again, there are major differences, which include the exquisite specificity of

antibodies for antigens versus the spreading of sensitivities to structurally unrelated chemicals that is reported by chemically sensitive patients.

In effect, chemical sensitivity is a sort of “cryptotoxicity.” “*Crypto*” (Gk. “hidden”) because of:

(1) The frequently hidden or imperceptible nature of the initial exposure(s) that causes loss of specific tolerance. This event may go unnoticed entirely or may not be connected causally with onset of illness by patient or physician. For example, an illness that develops following routine household extermination or administration of a general anesthetic may be attributed to some other cause. If several residents of a home recently treated with pesticides develop flu-like symptoms at the same time, an infectious cause may be assumed erroneously.

(2) The diversity of clinical presentations that occur, even among those who share the same inducing exposure event(s) (e.g. identically or near-identically exposed family members or co-workers). Such symptom heterogeneity in an exposed group would tend to “hide” the presence of a discrete illness, thus thwarting standard epidemiological approaches to investigating an outbreak.

(3) Masking via acclimatization, apposition and addiction, which have been described previously. Following loss of specific tolerance, masking obfuscates the relationship between symptoms and triggering exposures.

The term “toxicant-induced loss of tolerance” (acronym “TILT”) might describe this cryptotoxic theory of disease more parsimoniously. An analogy comes to mind here: with a pinball machine, a player has just so much latitude—he can jiggle the machine, nudge it, bump it, rock it, but when he exceeds the limit for that machine, the “TILT” message appears, the lights go out, and the ball cascades to the bottom. The machine’s tolerance has been exceeded and no amount of effort will make the bumpers or flippers operate as they did before. The game is over. A colleague recently suggested that another reason a pinball analogy was apt was the fact so many MCS patients bounce around from one specialist to another. Indeed the average MCS

patient in one study had consulted nearly ten physicians (Miller and Mitzel, 1995).

Is *another* name for chemical sensitivity really necessary? Notably, the term “allergy” might have sufficed. Patients with chemical sensitivities spontaneously refer to their responses as “allergies.” Indeed, when Von Pirquet coined the word “allergy” in 1906, he defined it as “altered reactivity” of *whatever* origin. The term originally embraced both immunity and hypersensitivity. In 1925, European allergists influenced their American counterparts to redefine allergy in the context of antibodies and antigens. A few American allergists objected, observing that hypersensitivity in some patients could be occurring on a non-immunologic basis. In 1967, the discovery of IgE cemented the antibody-antigen definition of allergy into place, apparently permanently. Allergists have spent many hours instructing the MCS patients who consult them that what they have is not an allergy.

8. Association versus causation

Critics of MCS correctly observe that “humans have a desire to assign a cause for everything.” The history of mankind is filled with examples of man’s attempts to assign cause to every event, particularly to illness, misfortune, or death (Waddell, 1993). The question is, how do we distinguish between a chance association and true cause and effect? Sir Austin Bradford Hill (1965) offered nine criteria that have been widely used by epidemiologists to help make this distinction: (1) Strength of the association, i.e. between the exposure and the illness. For example, Percival Pott observed an enormous, perhaps 200-fold increase in scrotal cancer among chimney sweeps versus workers not exposed to tar or mineral oils, a strong association indeed. However, Hill cautions, we should not be too ready to dismiss a cause-and-effect hypothesis merely on the grounds that the observed association appears slight, because there are many instances in medicine when this occurs, yet a cause-and-effect relationship exists. For example, only a few persons who harbor meningococcus develop meningitis

from it, and only a few individuals who are stung by bees develop anaphylaxis. Analogously, only a few persons exposed to certain pesticides or a sick building appear to develop MCS.

(2) Consistency. Have different people in different places and times observed the association? Hill considers this especially important for rare hazards or conditions. With regard to chemical sensitivity, multiple observers have independently described chemical sensitivity arising in persons exposed to organophosphate pesticides (Rosenthal and Cameron, 1991; Cone and Sult, 1992; Miller and Mitzel, 1995).

(3) Specificity of the association. The more the association is limited to specific exposures and/or to specific types of disease, the clearer the case for causation. Research on inducing exposures for MCS might reveal strong, specific associations. With respect to triggering, there appears to be lack of specificity both in terms of exposures and symptoms. However *individual* MCS patients report specific symptoms with specific exposures. Unlike for cancer or heart disease, cause and effect for symptom triggering in MCS can be tested experimentally in humans, providing direct experimental measurement of the specificity of the association (if it exists), the strongest form of evidence possible for an environmentally-related illness.

(4) Temporality. Does the exposure precede the illness? Some authors have noted depression or somatoform tendencies in some MCS patients that preceded their "initiating" exposure event. Perhaps the strongest evidence for temporality is the temporal cohesiveness between exposure and onset of symptoms that has been observed in large exposure groups, for example, the Environmental Protection Agency's sick building occupants or the Gulf veterans, many of whom report new-onset intolerances and have no evidence of psychiatric problems predating their exposure.

(5) Biological gradient. An association that follows a biological gradient or dose-response curve strongly suggests causality. Hill acknowledges that it is frequently difficult to obtain a satisfactory measure of exposure. However, a dose-response relationship that has been inferred for allergic conditions (Waddell, 1993) may also per-

tain to chemical sensitivity: there is a dose-response relationship for the first, sensitizing exposure in a susceptible individual; with subsequent exposures, the now sensitized person also responds in proportion to the dose, but at a much lower dose level. In addition, MCS patients frequently report that the longer they remain in an exposure situation, the more severe their symptoms become and the longer they persist. Again, in contrast with cancer or other environmentally-related diseases, the triggering phase of chemical sensitivity lends itself to direct human testing of a dose-response relationship, thus obviating the need for speculation about a biological gradient. (6) Plausibility. Hill comments that it is helpful if the causation we suspect is biologically plausible, but that *what is plausible depends upon the biological knowledge of the time*: "in short, the association we observe may be new to science or medicine and we must not dismiss it too lightheartedly as just too odd." In fact there are some medical conditions which have features that are strikingly similar to MCS and which are well-accepted, for example, Reactive Airways Dysfunction Syndrome (discussed previously) and Multiple Drug Allergy Syndrome (Sullivan, 1991). These parallel clinical observations may be signs pointing in the direction of biological plausibility for MCS.

(7) Coherence. The cause-and-effect relationship under scrutiny should not conflict with other generally known facts about the disease, e.g. the pathology or biochemistry of the illness. Since so little research has been done on MCS, so far this has not been a problem.

(8) Experiment. Experimental evidence can provide the strongest support for a cause-and-effect relationship. Perhaps one of the reasons MCS patients are so dogged in their insistence that chemicals are causing their symptoms is the strength of the experimental evidence they perceive when they deliberately avoid and then are re-exposed to incitants. Part of the appeal of MCS, at least to some environmental scientists, is that it poses an experimentally testable hypothesis, in contrast with most other environmentally-related illnesses of major concern. But, again, experimental conditions must be optimized, i.e.

unmasking in an environmentally-controlled hospital unit, if the most robust effect is to be seen. Currently, the *only* obstacle to these studies being undertaken is lack of funding.

(9) Analogy. Under certain circumstances, cause-and-effect can be inferred by analogy. The sensitivities reported by MCS patients are reminiscent of the heightened sensitivity to tobacco smoke reported by those who have recently quit smoking. Likewise, there are close parallels between MCS and addiction, in which food cravings and bingeing are also reported. MCS patients describe "going through withdrawal" or "detox" during which the symptoms they report are reminiscent of those reported by drug abusers, yet most MCS patients systematically avoid even mildly addictive substances. Other possible analogues to MCS are Reactive Airways Dysfunction Syndrome (RADS) and toluene diisocyanate (TDI) sensitivity, particularly the former in which a single major exposure may lead to hyperresponsiveness to multiple, chemically-unrelated inhalants. We must ask ourselves, if the airways can develop sensitivity to multiple chemicals, by analogy why couldn't the central nervous system do so as well?

To Hill's criteria, I might add a tenth criterion, one that would apply to symptoms (or illnesses) that are primarily subjective in nature:

(10) Unique symptomatology. The more obscure or unique a symptom is, particularly if it is reported by several independent exposure groups (for example, industrial workers, white collar professionals, Gulf veterans), the greater the likelihood of causation. For MCS, it would be difficult to imagine that the curious symptom of odor intolerance, which has been reported by demographically diverse groups following various exposure events, could be "invented" by all of them. Equally unexpected and counter-intuitive are MCS patients' practices of avoiding fragrances, foods, alcoholic beverages, etc., that they formerly relished. Why would anyone who really liked pizza, chocolate or beer give them up unless they made them ill? Why would a mechanic who loved his job and used to think that WD-40 would make a wonderful perfume, suddenly report that odors at work made him ill, if in fact they did

not? Why would doctors, lawyers, teachers and others say they quit their professions because of severe mental confusion around fragrances and engine exhaust, if this weren't the case? Scientifically, it would be absurd to dismiss such eccentric behaviors in otherwise sane individuals without searching exhaustively for a plausible biological basis.

Hill suggests that his criteria be used to "study association before we cry causation." He further cautions that none of the criteria indisputably revokes a cause-and-effect hypothesis, and none is a *sine qua non*. In aggregate, these criteria assist in determining causation. As discussed previously, chemical sensitivity appears to involve two steps: Induction by a major or repeated exposure, and (2) subsequent triggering of symptoms by chemically-unrelated, low level exposures. Verification of the second of these two steps lends itself to direct experimental testing. Validation of the first step may rest upon epidemiological investigations and animal studies.

9. Conclusion

If we now begin to speak of chemical sensitivity—the theory of disease, instead of chemical sensitivity—the symptom or the syndrome, the types and number of individuals potentially affected by the condition shift dramatically. While roughly one-third of the population reports the *symptom* of chemical sensitivity, only a fraction of these individuals suffers from a *syndrome* that disrupts their health or lifestyle. In contrast, chemical sensitivity—the *theory of disease* posits that familiar, chronic conditions like depression, migraine, fatigue, and asthma can arise from environmental exposure. The health care costs associated with these conditions are undeniably enormous. Notably, many of these same conditions also appear to be becoming more prevalent, which should further pique our curiosity about environmental causation, particularly given the exponential increases in synthetic organic chemical and pesticide production that have taken place in this country since World War II, coupled

with the fact that most Americans spend 90% or more of their day indoors, often in tightly constructed buildings with poor air quality.

How we define and conceptualize chemical sensitivity drastically affects our perspective regarding the seriousness of "low level" environmental exposures. As a symptom, chemical sensitivity or pathosmia generally is not disabling; as a syndrome, multiple chemical sensitivity is uncommon (although if only 1% of Americans were affected, it would still amount to a few million people). Those who see chemical sensitivity as an emerging theory for disease see something profoundly different and deeply concerning.

Only carefully designed studies in a controlled environment will answer the complex questions before us concerning the environmental etiologies of many chronic diseases. Studies of this kind will be essential for determining what role toxicant-induced loss of tolerance plays, if any, in human disease. From the standpoint of public health, if it were to be determined that chemical sensitivity was limited to a small group of vulnerable individuals in the population, perhaps strategies for protecting or accommodating them could be devised, such as special ventilation, working at home, wearing respirators, etc. Indeed such things already are being done. On the other hand, if chemical sensitivity is an emerging theory of disease that applies not only to MCS, but also to depression, migraine, asthma, chronic fatigue syndrome, illnesses associated with implants, and other conditions, the stakes go up considerably. Medical care costs in this nation have risen from 5.3% of gross domestic product in 1960 to 13.9% in 1993, with a dollar value exceeding \$1 trillion, nearly \$4000 per person (Grumbach and Bodenheimer, 1994; Levit et al., 1994). An important question is, how much does toxicant-induced loss of tolerance contribute to this sum?

These are vexing questions that go against the grain of accepted explanations concerning the origins of illness. In recent times, many chronic diseases, addiction, and violence have been explained in whole or in part in terms of the psyche and stress. An enormous amount of research has been devoted to these explanations. There are

about 37 000 psychiatrists and 241 000 psychologists in the United States (Roback et al., 1994; Statistical Abstract of the United States, 1994). Any theory of disease so bold as to suggest that depression, anxiety, panic attacks or fatigue might be caused by chemical exposures should expect a less than enthusiastic reception. Yet, most would agree *in principle* that organic bases for illnesses should be ruled out before psychological explanations are invoked. In medicine, millions of dollars are devoted to the study of the chronic conditions discussed here and to identifying psychological therapies and drug interventions. Yet, *what if* such therapies alleviated some but not all symptoms, and in the meantime causative exposures were overlooked? Such a notion seems farfetched, yet it is precisely what some heretical scientists and physicians now propose. A skeptical regard for such speculation by most practitioners should be viewed as a rational response to the flurry of pronouncements and papers of varying reliability that have appeared on chemical sensitivity in recent years. Wherever the truth lies, the costs of not pursuing scientific resolution of these questions are potentially enormous.

Perspective is important. Finding a biological marker for chemical sensitivity may prove as difficult as finding the bacterium that was causing cholera or identifying IgE in allergic diseases. Generally speaking, treatment and intervention need not await the discovery of a marker. Hand washing, sanitation, and allergy shots were all introduced based on clinical observations. Physicians over the centuries have had to tolerate uncertainty and cautiously adopt new therapies while awaiting scientific explanations, but never before in a nation or time with such high-tech medical prowess and vested corporate interests, neither of which has much enthusiasm nor patience for chemical sensitivity.

Science is not about belief. Science is about "guess and test." A new "guess," perhaps an emerging theory of disease that parallels the germ theory, has come to our attention. The theory suggests that certain costly, chronic illnesses may be caused by familiar exposures. The theory insinuates that environmental exposures may

cause loss of tolerance for specific chemicals, foods and drugs, that is, toxicant-induced loss of tolerance. The question is, shall we expend scant resources to explore this theory, or will we continue the present debate?

"When I use a word," Humpty Dumpty said, in rather a scornful tone, "it means just what I choose it to mean—neither more nor less."

"The question is," said Alice, "whether you can make words mean so many different things."

"The question is," said Humpty Dumpty, "which is to be master — that's all."

Lewis Carroll (emphasis added)

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ELSEVIER

Toxicology 111 (1996) 87-100

TOXICOLOGY

Sensitization induced by kindling and kindling-related phenomena as a model for Multiple Chemical Sensitivity

John Rossi III

*Naval Medical Research Institute Detachment (Toxicology), Tri-Service Toxicology Consortium, 2612 Fifth St., Area B,
Wright-Patterson AFB, OH 45433-7903, USA*

Abstract

It has been suggested that the neurobehavioral dysfunction observed in persons presenting with symptoms of Multiple Chemical Sensitivity (MCS) syndrome involves sensitization of neural circuits. Two hypotheses for the route of exposure in induction of neural sensitization in MCS are: (a) direct chemical stimulation of olfactory processes, or (b) general systemic response to inhaled chemicals. In either case, the mechanism of action may involve chemical kindling or kindling-related phenomena. A neural sensitization mechanism based on kindling or kindling-related phenomena is attractive and has been previously demonstrated in both *in vitro* and *in vivo* animal models. Without a testable animal model for chemically mediated induction of MCS, however, any argument that MCS is mediated by kindling or kindling-related phenomena is reduced to the circular argument "the mechanism of sensitization is sensitization." The present survey provides an overview of the experimental paradigms that result in sensitization, differentiated on the basis of probable neurophysiological and neurochemical mechanisms. Neurophysiological potentiation, electrical kindling, chemical kindling and behavioral sensitization are evaluated and discussed in relationship to MCS.

Keywords: Multiple Chemical Sensitivity (MCS); Kindling; Neural sensitization; Behavioral sensitization; Neural potentiation; Animal models

1. Introduction

Multiple Chemical Sensitivity (MCS) has been described as a polysymptomatic syndrome characterized by neurobehavioral and physiological symptoms which include difficulties in concentration, fatigue, depression, headache, irritability, nausea, dizziness and sleep disorders (Randolph, 1978; Ashford and Miller, 1991; Miller, 1994). Ashford and Miller (1991) described a two-stage

process in the development of MCS. The first stage involves sensitization to an initial chemical exposure, while the second stage involves the triggering of reactions to subsequent chemical exposures. Individuals who acquire MCS may be initially susceptible by reason of genetics, hormones, nutrition, environment or stress. Prior to the sensitizing exposure, these persons generally lead normal lives and remain asymptomatic until the occurrence of the sensitizing event. The sen-

sensitizing event can be either a single high-level exposure, as might occur near a chemical spill, or repeated exposures to one or more chemicals at more moderate levels, as can occur from working in a new or newly renovated office building. The initial exposure may be sufficient to induce the "sensitized stage." Once sensitized, the individuals may adversely respond to other chemical triggers including low level exposures to smoke or perfume; drug and food/drug combinations, including alcohol and caffeine; and foods and/or food additives. Prior to sensitization, these individuals typically do not respond adversely to any of the trigger chemicals, and in many incidences (e.g. caffeine and ethanol) ingested them for pleasure. Repeated triggering may lead to further sensitization, with this progressive sensitivity spreading to numerous other compounds. Ashford and Miller (1991) define two symptomatic stages in MCS: (a) an adapted (masked) state wherein responses to exposures overlap in time so that relationships between symptoms and exposures are obscured; and (b) a de-adapted state where the relationships between symptoms and exposures are evident. As the severity of the symptoms increases, afflicted individuals may become incapacitated.

An olfactory-limbic model for the MCS syndrome was initially proposed by Bell et al. (1992). The interactive biopsychosocial model that they described includes provisions for susceptibility of populations at risk (i.e. genetic, hormonal, nutritional or stress induced predisposition), as well as a neural model for the development of the chemical sensitivity. The initial model proposed that some combination of direct olfactory stimulation (e.g. Bokina et al., 1976), systemic chemical kindling (e.g. Mason and Cooper, 1972; Post et al., 1975; Post and Kopanda, 1976), partial kindling (e.g. Adamec and Stark-Adamec, 1983) and time-dependent sensitization (e.g. Antelman, 1988) could account for the initiation, amplification and persistence stages of the MCS. Further refinements of the neural sensitization model (Bell, 1994; Bell et al., 1993a,b,c, 1994) focused on time-dependent sensitization (TDS) and partial limbic kindling as mediators of the syndrome.

All versions of the neural sensitization model

implicate neural sensitization mechanisms in the ontogeny of MCS, and Bell (1996) provides convincing face, construct and criterion validation evidence for the involvement of neural sensitization in cacostmia and MCS. The specific mechanisms that might be involved, however, are alluded to only allegorically in terms of TDS, partial kindling and kindling-related phenomena. In order to develop a comprehensive model useful in the study of MCS and related disorders, it is necessary to elaborate on the specific nature of the neural sensitization mechanism(s) that might be involved. The present paper provides an overview of the paradigms used to induce and demonstrate neural sensitization and evaluates the likelihood of their involvement in MCS.

2. Direct neural sensitization

2.1. Post-tetanic potentiation (PTP)

The earliest demonstration of direct neural sensitization was made by Larrabee and Bronk (1947) in experiments investigating prolonged facilitation of synaptic excitation after repeated stimulation in the stellate ganglion. The phenomenon, known as post-tetanic potentiation (PTP) or post-activation potentiation, results from repeated stimulation (tetanization) of a monosynaptic pathway or nucleus giving rise to a monosynaptic pathway. PTP appears to be a generally exhibited property of nervous tissue (see Hughes, 1958, for an extensive review). PTP has been observed in mammalian neural tissue at the neuromuscular junction, in sympathetic ganglia, in spinal cord, in single neurons, and in sensory systems (Hughes, 1958). PTP has even been observed in the neuroblastoma cell line HT4 after direct neurotransmitter tetanization (Morimoto and Koshland, 1991).

The stimulation parameters required to induce PTP are varied, with tetanization frequencies ranging from 20 to 650 Hz, and with stimulation duration ranging from 5 to 30 s (Hughes, 1958). Sensitization in PTP is reflected in a statistically reliable increase from baseline in the amplitude of the evoked response at the terminal recording site. The duration of sensitization is typically

measured in minutes, although for some preparations the effect can persist for several hours (Hughes, 1958; Beswick and Conroy, 1965). PTP appears to be a presynaptic phenomenon through which an increased amount of neurotransmitter is released in tetanized neurons, mediated by changing neurotransmitter release dynamics (Esplin and Zablocka, 1969).

2.2. Long-term potentiation (LTP)

Similar to PTP, long-term potentiation (LTP) also involves repetitive stimulation of a monosynaptic pathway or nucleus giving rise to a monosynaptic pathway. First reported by Lomo (1966), LTP has become synonymous with the descriptive work of Bliss and Lomo (1973) and Bliss and Gardner-Medwin (1973) performed on potentiation of synaptic transmission in the hippocampus after stimulation of the perforant path in anesthetized and unanesthetized rabbits (for recent reviews of LTP see Tyler and DiScenna, 1987; Massicotte and Baudry, 1991). LTP has been described most thoroughly for the perforant path into the dentate area of the hippocampus, although it has also been reported for other forebrain structures (Massicotte and Baudry, 1991). LTP has been observed in *in vivo* preparations as well as in *in vitro* tissue slice preparations (Tyler and DiScenna, 1987; Schwartzkroin and Wester, 1975).

The stimulation parameters required to induce LTP are more complicated than those for PTP. Where PTP tetanizing stimulation is generally massed, LTP stimulation is generally delivered in pulse trains. Train length, stimulation current frequency, inter-train interval and total number of trains are highly variable over various experimental preparations. Similar to the stimulation that induces PTP, current amplitudes insufficient to generate epileptiform afterdischarge at the site of stimulation, yet sufficient to produce an evoked response at the terminal recording site, will yield LTP (Massicotte and Baudry, 1991). LTP generally persists much longer than PTP, with potentiation duration measured in hours and sometimes days (Massicotte and Baudry, 1991). A further distinction between PTP and

LTP can be made on the basis of mediation of the potentiation. As previously discussed, PTP appears to be a solely presynaptic phenomenon dependent on transmitter release dynamics, while LTP may depend on increased transmitter efficiency as well as sensitization of post-synaptic processes (Bliss and Lomo, 1973).

3. Kindling

3.1. Electrical kindling

The term electrical kindling refers to the induction of generalized epileptic seizures following repeated electrical stimulation of brain tissue at levels initially insufficient to induce motor convulsions. Watanabe (1936) first observed that daily cortical stimulation of freely moving dogs led to a progressive development of motor seizure activity, eventually resulting in spontaneous episodes of *status epilepticus*. The phenomenon was not extensively studied until Goddard (1967) recognized its potential importance, and Goddard et al. (1969) reported the seminal experiments documenting long-term sensitization induced by the kindling effect. These researchers established that susceptibility to kindling is a general property of neural brain tissue. They demonstrated that the effect could be elicited by stimulating a large number of different brain regions, although some brain areas required considerably more stimulations to induce generalized seizures than others. These and subsequent studies suggest that the most reliable subcortical sites for kindling are structures in the limbic system or those areas with efferent projections to limbic structures, including the olfactory system (Cain, 1977). Goddard et al. (1969) also determined that interstimulation interval was relatively unimportant, showing that the number of stimulations required to achieve a generalized seizure was similar regardless of whether the interval between stimulations was 24 h or 7 days. They did, however, report that more stimulations were required when the interstimulation interval was 12 h or less, and that kindling of generalized seizures did not occur when the interstimulation interval was less than 20 min. Finally, Goddard

et al. (1969) demonstrated that sensitization induced by kindling does not substantially weaken over a period of 3 months, even if no further electrical stimulations are delivered. Racine (1978) stated that amygdaloid-kindled rats remain sensitive to the kindling stimulation for periods as long as 14 months.

The interspecies generality of the kindling phenomenon has been demonstrated repeatedly. Electrical kindling has been observed in the baboon (Wada and Osawa, 1976), the monkey (Goddard et al., 1969), the cat (Tanaka, 1977), the rabbit (Tanaka, 1972), the mouse (Leech and McIntyre, 1976) and the frog (Morrel and Tsuru, 1976). In mammalian studies, kindling induction patterns are typically very similar, although inter-species differences exist regarding the number of afterdischarge-eliciting stimulations necessary to achieve kindled motor seizures. For mammalian species, kindling susceptibility appears to be inversely related to phylogenetic ranking (e.g. cats kindle more slowly than rats but more quickly than monkeys).

Racine (1972a,b) was the first to study the electrographic events that occur during the kindling process. Racine (1972b) demonstrated that kindling of motor seizures requires the generation of afterdischarges in the area stimulated by the stimulating current. Throughout the kindling process, brain areas increasingly distal to the stimulating electrode are, with successive stimulations, progressively recruited to exhibit epileptiform afterdischarge. Behavioral seizure indices progress with distal tissue recruitment (i.e. facial twitching, then forequarter jerking, then forelimb clonus, etc.), culminating in a generalized motor seizure. Racine (1972a) demonstrated that the threshold current for elicitation of afterdischarge could be lowered by repeated stimulation with current amplitudes initially insufficient to evoke afterdischarges, and the reduction in afterdischarge threshold observed was found to be relatively permanent. The importance of the afterdischarge waveform in the processes which underlie neural recruitment, and of the concomitant progressive development of motor seizures, was highlighted by the subsequent finding that single trial motor seizures could be elicited by

stimulating current that mimicked afterdischarge potentials in terms of frequency (Racine et al., 1973).

The physiological substrates of electrical kindling appear to be related to those that underlie both PTP and LTP, although this relationship is not unambiguous (Cain, 1989b). Single-pulse stimulations in a kindled amygdala have been shown to facilitate evoked potentials in target structures. This effect is similar to that observed after stimulations that induce LTP (Racine et al., 1975, 1983), although the persistence of the kindled response may be significantly longer than with LTP. Failure to observe a kindling-induced effect on evoked potentials (Giacchino et al., 1984), and the converse finding of decreases in evoked potential amplitude have also been reported (Racine et al., 1983; Sutula and Steward, 1987), although these inconsistencies may be explained by changes in evoked response thresholds that may occur during kindling. Indeed, Maru and Goddard (1987) demonstrated that kindling-induced enhancement of the evoked response is maintained throughout the kindling process if the amplitude of the single-pulse stimulation current is increased to compensate for the increasing thresholds of the evoked response. Similarly, while LTP-inducing stimulation has been demonstrated to facilitate kindling (Racine et al., 1975a; Sutula and Steward, 1987), the actual facilitation never exceeds 50% savings in number of afterdischarge-eliciting stimulations required for behavioral convulsions. Additionally, repeated LTP-inducing stimulations that do not trigger afterdischarges fail to kindle motor seizures or lower the threshold for eliciting afterdischarges (Sutula and Steward, 1987). A consensus appears to be forming (Jibiki et al., 1988; Cain et al., 1992) to support the hypothesis that the underlying substrates of LTP may also underlie the establishment of kindling, but not necessarily its persistence. Furthermore, it is believed that the mechanism underlying sensitization induced by either LTP or kindling procedures may involve activation of the *N*-methyl-D-aspartate (NMDA) receptor (Cain, 1989a; Gilbert and Mack, 1990; Cain et al., 1992).

3.2. Partial kindling

A variant of the electrical kindling paradigm has been referred to as partial kindling (Adamec and Stark-Adamec, 1983). As with electrical kindling, partial kindling induction requires the elicitation of epileptiform afterdischarges, although the stimulating electrode is generally located in a brain nucleus or area implicated as being involved in specific behavioral responding. As in electrical kindling, the afterdischarge duration increases with successive stimulations and requires a minimum interstimulation interval. Accordingly, areas more distal to the electrode site are progressively recruited to exhibit epileptiform afterdischarge with successive stimulations. Unlike the classical electrical kindling paradigm, however, partial kindling is discontinued before the elicitation of generalized motor seizures. The dependent variables in partial kindling experiments are a change in some behavioral endpoint and the persistence of the change.

The use of what is now referred to as partial kindling was pioneered by Jose Delgado and his co-workers in the 1950s. Delgado and Anand (1953) reported a long lasting increase in food intake after repeated spaced stimulations of the lateral hypothalamus of the cat. This procedure appears to mimic the functional effects of continuous lateral hypothalamic stimulation that has been shown to increase feeding behavior in animals (Berridge and Valenstein, 1991). Similarly, Alonso-DeFlorida and Delgado (1958) showed that repeated amygdala stimulations result in persistent alterations in the social behavior in the cat. More recently, Adamec (1990, 1991) has used a partial kindling procedure to study aggressiveness in cats, reporting that partial kindling of the amygdalofugal pathway permanently alters social behavior of the animals.

4. Chemical kindling

Chemical kindling refers to progressive induction of generalized motor seizures following repeated administration of chemical compounds at dose levels initially insufficient to induce motor convulsions. Unlike electrical kindling, in which a standard kindling procedure is used to achieve

the desired endpoint (i.e. either generalized motor seizures or relatively permanent changes in specified behaviors), chemical kindling refers to a much broader assortment of paradigms. The convulsive endpoints of the chemical kindling paradigms may not have common underlying mechanisms and may not be unambiguously related to each other or to those induced by electrical kindling. Several classes of chemical kindling can be readily differentiated on the basis of the procedures used in induction of the generalized seizures, as well their likely mechanisms of action.

4.1. Intracerebral-localized (ICL) chemical kindling

Initially described by Goddard (1969), intracerebral-localized (ICL) chemical kindling is most paradigmatically similar to classical electrical kindling among the chemical kindling procedures. In ICL chemical kindling, a convulsant, neurotransmitter, or receptor agonist or antagonist is administered directly to brain tissue through a narrow diameter cannula. The kindling agent is infused periodically, generally at a frequency of once per day. Similar to electrical kindling, the infused stimulus must elicit localized epileptiform afterdischarges. Subsequent infusions increase the duration of the afterdischarge and lead to recruitment of brain regions more distal to the infusion site. A gradual progression to generalized motor seizures is observed. ICL chemical kindling has been demonstrated in response to intracerebral infusions of the cholinomimetic agent carbachol (Goddard, 1969; Vosu and Wise, 1975), the opioid agonist methionine-enkephalin (Tanaka et al., 1993), the γ -aminobutyric acid (GABA) antagonist picrotoxin (Cain, 1987), the GABA antagonist bicuculline methiodide (Uemura and Kimura, 1988), the convulsant pentylenetetrazol (Cain, 1982) and the cyclic nucleotide cAMP (Yokoyama et al., 1989).

4.2. Intracerebroventricular (ICV) chemical kindling

Intracerebroventricular (ICV) chemical kindling has also been demonstrated (Snead, 1983). ICV chemical kindling is similar to localized

(ICL) chemical kindling in that a progression to generalized motor seizures is observed after spaced repeated ICV administrations of compounds that also support ICL chemical kindling. ICV chemical kindling differs from ICL chemical kindling in that the kindling compounds are infused into the cerebral ventricles and achieve a much broader dispersion. This dispersion property suggests that ICV chemical kindling may be more closely related to systemic chemical kindling (as described below) than to electrical kindling. As in systemic chemical kindling, the wide distribution of the convulsant compound makes it difficult to specify the locus or mechanism of action of the chemical compound accurately. ICV kindling has been demonstrated after repeated administration of carbachol, morphine and leucine-enkephalin (Snead, 1983).

4.3. Systemic chemical kindling

Systemic chemical kindling refers to a gradual progression to generalized motor seizures observed after spaced, repeated intraperitoneal (i.p.), intravenous or subcutaneous administrations of a chemical compound. Downs and Eddy (1932) provided the first convincing demonstration of this effect. They reported that repeated i.p. injections of cocaine, separated by up to 3 days, led to severe convulsions and death in rats. The first report of systemic chemical kindling in the post-Goddard electrical kindling literature was by Mason and Cooper (1972). They demonstrated that initially non-convulsive doses of pentylenetetrazol (PTZ), were subsequently capable of inducing generalized motor seizures when repeatedly administered at 48-h intervals. More recently, these results have been repeated using other known convulsant compounds including: picrotoxin and bicuculline (Nutt et al., 1982b; Nutt et al., 1982a); the β -carboline compounds Ro 15-1788 (Morin et al., 1983; Morin, 1984) and FG-7142 (Little et al., 1986); and the pesticide endosulfan (Gilbert, 1992). Racine has concluded that the capability to induce systemic chemical kindling is a general property of convulsant compounds (Racine, 1978).

Cocaine, a releaser and reuptake inhibitor of the catecholamine neurotransmitters norepineph-

rine and dopamine (Cooper et al., 1986), has been found to support systemic chemical kindling (Post and Kopanda, 1975). This finding is paradoxical to the accepted belief that catecholamine neurotransmitters generally inhibit the kindling process (Corcoran, 1981). Accordingly, it has been suggested that the chemical kindling effect of cocaine is due to properties associated with its local anesthetic action. This conclusion is supported by the demonstration of similar chemical kindling after repeated injections of the local anesthetic lidocaine (Post et al., 1975; Post and Kopanda, 1976), and the finding that paroxysmal EEG activity accompanies generalized motor seizures after very high doses of the compound (Riblet and Tuttle, 1970). However, failure (Rossi, unpublished observation) to replicate the kindling effect in Long-Evans rats after 28 daily injections of the local anesthetic procaine hydrochloride (50 mg/kg) suggests that kindling is not necessarily a general property of local anesthetics.

A flaw in the majority of studies inducing systemic chemical kindling is the lack of report of EEG correlates to the behaviors induced by the chemical administration. Indeed, Racine (1972a,b) has pointed out that many of Goddard's initial observations of low electrical kindling rates, or failure to observe kindling at some electrode placements, can be explained by use of current amplitudes insufficient to induce the afterdischarges necessary to support kindling. In spite of the knowledge of this pitfall, most chemical kindling studies evaluate seizure activity by exclusively assessing behavioral convulsive responses according to some variant of the five-stage scale developed by Racine (1972b). Drawing firm conclusions from such studies is made difficult by the lack of knowledge of the electrographic dose-response characteristics of the pharmacological manipulations.

Only two studies found in the literature examined EEG as a function of the administration of a convulsant in a systemic chemical kindling study. Ito et al. (1977) measured cortical EEG during a PTZ kindling procedure and assessed both EEG and behavioral responses. Ono et al. (1990) correlated behavioral and EEG responses

during a systemic chemical kindling procedure, also using PTZ. Ono et al. developed a correlative rating system allowing extrapolation of the electroencephalographic state of the brain based on the behavioral observations. The result of this investigation was a six-stage seizure grading scale with EEG referents.

Two shortcomings of this behavior-to-EEG work are obvious. First, the only compound to be systematically studied with both EEG and behavioral seizure indices is PTZ. Little is known about the electroencephalographic effects of other systemically administered compounds demonstrated to support chemical kindling. Second, while an ascending grading scale of seizure severity may be appropriate for categorization of motor activity associated with electrical kindling or ICL chemical kindling, use of such scales may not be appropriate for the grading of seizures elicited by systemic chemical stimulation. Indeed, the nature of electrical and ICL chemical kindling allows description of a well-defined primary focus, description of pathways supporting consequent recruitment and epileptogenesis, evaluation of propagation strength, and observations of convulsive motor behavior correlated with the activity at the primary site. In such kindling, a systematic progression of severity of behavioral response is observed as a consequence of recruitment of distal brain areas involved with motor activity. Specification of localization is more difficult using systemic or ICV chemical stimulation because many structures are simultaneously activated by effective doses of the chemical. Repeated administrations of chemical convulsants (e.g. systemic PTZ) do not necessarily mimic the structure-to-structure recruitment effects seen in electrical or ICL chemical kindling, but more likely produce regionally graded changes in epileptiform discharge patterns in areas related to motor behavior. While electrical kindling appears to be the result of recruitment to epileptic involvement of distal brain areas related to motor activity, systemic chemical kindling appears to reflect changes in sensitization of all areas affected by the chemical. Electrical kindling and systemic chemical kindling may reflect two entirely different sensitization mechanisms, a possi-

bility suggested by the findings of a lack of differential responses to electrical stimulation in brains of mice previously kindled with PTZ (Piredda et al., 1986).

4.4. Chemical kindling of EEG paroxysmal activity without behavioral convulsions

In the process of describing the convulsant actions of a novel organophosphate compound trimethylolpropane phosphate (TMPP), we have uncovered a heretofore unreported form of non-convulsive, chemical kindling of EEG. TMPP reliably induced EEG paroxysms in rats at i.p. doses as low as 0.025 mg/kg, and induced generalized behavioral convulsions progressing to *status epilepticus* and death at doses greater than 0.4 mg/kg, indicating that it possesses about 100 times the convulsive potency of PTZ (Ono et al., 1990). Rats from both Fischer-344 and Sprague-Dawley strains underwent a standard tri-weekly systemic chemical kindling procedure using TMPP doses from 0.10 mg/kg to 0.40 mg/kg. Mid-range doses of TMPP (0.20-0.25) were found to induce brief episodes of head myoclonus in several animals after the first injection, while the highest dose (0.40 mg/kg) induced generalized motor seizures in all animals tested. No progressive changes occurred in behavioral convulsive activity observed during the 11-week kindling procedure, however, regardless of the dose level used. The group that was kindled with the 0.40 mg/kg dose exhibited erratic patterns of occurrence of generalized seizures, whereby animals that exhibited generalized motor seizures in response to the initial TMPP injection sometimes exhibited no behavioral convulsive response to subsequent administrations. These failures to exhibit a generalized motor convulsion were found to become more frequent as the 10-week kindling procedure progressed. Remarkably, while behavioral indices of kindling remained constant or declined, EEG paroxysmal activity showed a significant increase in response to repeated exposure to the same dose level of TMPP. Although TMPP has been shown to be >99% cleared from major body tissues within 24 h of administration, the pattern of increased EEG paroxysmal activity (over baseline levels) persisted 2 weeks

following the last TMPP administration and fell to baseline level during the ensuing week. The animals that underwent the kindling procedure were also found to exhibit long-term sensitization to an auditory stimulus when tested for susceptibility to audiogenic seizures 74 days following the last TMPP injection. A comprehensive description of the mechanisms of action of TMPP may prove paramount in the elucidation of the processes subserving neural sensitization.

5. Sensitization induced by drugs, toxicants or stressors

5.1. Long-term sensitization (LTS)

Long-term sensitization (LTS) refers to the processes underlying changes from baseline of some physiological or behavioral endpoint that are reflected shortly after the sensitizing event and which persist for an indefinite period of time. The general procedure for assessing LTS first involves assessing the baseline rate or level of a physiological or behavioral endpoint. Next, a sensitizing drug, toxicant, noxious stimulus or other stressor is administered to the experimental subject in one or more treatments. The physiological or behavioral endpoint is tested again, usually within 48 h of the sensitizing treatment. If a reliable change from baseline is measured, sensitization has occurred. Finally, the endpoint is tested again weeks or months after the sensitizing treatment, and if a reliable change from baseline is still observed, LTS has occurred. The unique property of LTS is that while it exhibits long-term persistence, it does not require time to develop.

Bartholetti et al. (1983) demonstrated LTS to the excitatory effects of morphine in dependent rats. Sensitization was found to persist for 160 days following initial post-sensitization testing. More recently, long term sensitization of apomorphine-induced rotational behavior has been described in striatally lesioned rats (Klug and Norman, 1993). In this study, rats exhibited short-term sensitization to a second administration of apomorphine administered 36 h after the initial sensitizing dose. LTS was observed 10-12 weeks following initial sensitization. We have

observed long-term physiological as well as behavioral sensitization to the effects of TMPP. We initially demonstrated that one TMPP administration at concentrations as low as 0.0125 mg/kg is capable of sensitizing Fischer-344 rats to audiogenic seizure induction 30 min following administration. These rats, as well as TMPP treated rats not initially tested for audiogenic seizure susceptibility, exhibited LTS to an audiogenic stimulus up to 80 days following TMPP administration. Subsequently, we demonstrated behavioral sensitization to the effects of d-amphetamine 2 days after four bi-daily administrations of TMPP (0.10 mg/kg), and LTS 61 days following the final TMPP treatment.

5.2. Time-dependent sensitization (TDS)

Time-dependent sensitization (TDS) is paradigmatically similar to LTS. It refers to the processes underlying changes from baseline levels of some physiological or behavioral endpoint that are not reflected immediately after the sensitizing event, but require a duration of many days for demonstration of sensitization. The general procedure for assessing TDS first involves measurement of the baseline rate or level of a physiological or behavioral endpoint. Next, a sensitizing drug, toxicant, noxious stimulus or stressor is administered to the experimental subject in one or more treatments. The physiological or behavioral endpoint is tested again, usually within 48 h of the sensitizing treatment. Testing within this interval must reveal no reliable change in the endpoint from baseline values. Finally, the endpoint is tested again weeks or months after the sensitizing treatment. If a reliable change from baseline is observed, TDS is said to have occurred. The unique property of TDS is that it requires many days to develop after experience with the sensitizing event.

The TDS paradigm has been thoroughly reviewed by Antelman (1988). TDS has been demonstrated for single injections of any of a number of psychoactive drugs. TDS of either physiological or behavioral responses has been reported for cocaine, amphetamine, amytryptaline, haloperidol, diazepam, opiates, ethanol and clonidine. Antelman et al. (1980) demonstrated that stres-

sors can act interchangeably with psychostimulants in the production of TDS of both behavioral and physiological responses. Sensitization was reported for physical stressors including tail pinch and needle prick (Antelman, 1988), as well as for emotional stressors (Antelman et al., 1992). Nocjar (1995) demonstrated psychostimulant and opiate induced TDS of both sexual and food driven appetitive behaviors using a novel open field testing procedure. In addition to behavioral and neurophysiological TDS effects, time-related effects have also been demonstrated for immunosuppression (Antelman et al., 1990), and for melatonin receptor down regulation with accompanying increases in adenylyl cyclase activity after treatment with melatonin (Hazlerigg et al., 1993).

6. Learning

Learning, as described in most introductory psychology textbooks as a relatively permanent change in behavior brought about by experience, clearly represents the most ubiquitous form of sensitization. Learning is also the most powerful demonstration of the adaptability of the central nervous system (CNS). If a continuum exists in sensitization, and the most simplistic physiological demonstrations of sensitization (i.e. as PTP and LTP) represent the lowest level, and kindling and the complex multifaceted demonstrations of LTS and TDS represent intermediate levels, then learning would represent the highest level in the continuum. In the cases of PTP and LTP, a rather short duration of sensitization is observed in monosynaptic circuits induced by a very simple electrical stimulus. In the cases of LTS and TDS, a long-term sensitization is observed in very complex circuits after exposure to a moderately complex pharmacological or traumatic stimulus. In the case of learning, a relatively permanent sensitization occurs in circuits of enormous potential complexity, in response to multisensory experiential information. Learning may actually represent an indirect index of physiological sensitization that is manifested in behavioral change. The main problem with assessing learning in the context of the sensitization

continuum is that, except for simple learning that occurs in the well-mapped nervous systems of annelid worms, insects or simple marine creatures, describing the physiological and biochemical changes that occur during the sensitization process with the technology available today is nearly impossible. From the time scientists first concluded that the substrates sensitized in the learning process exist in the neural tissue of the brain, the search for those substrates has remained an enigma (Lashley, 1950).

7. Sensitization and multiple chemical sensitivities

7.1. Kindling-like sensitization and MCS

The previous sections of this paper discussed well-known neurophysiological mechanisms that may, in part, explain development, manifestation and persistence of MCS. The concluding part of this paper hypothesizes how the previously described sensitization phenomena might relate to MCS and suggests a strategy for the development of a physiological model for MCS.

Ashford and Miller (1991) and Miller (1994) proposed that any model sufficient to explain MCS needs to address the following clinical observations:

- (1) Symptoms involving virtually any system in the body or several systems simultaneously, but most frequently the CNS (fatigue, mood changes, memory and concentration difficulties);
- (2) Different symptoms and severity in different individuals, even among those experiencing the same exposure;
- (3) Induction or sensitization by a wide range of environmental agents, including pesticides, solvents and combustion products;
- (4) Subsequent triggering by lower levels of exposure than those involved in the initial induction of the illness;
- (5) Spreading of sensitivity to other, often chemically dissimilar substances. Each substance must trigger a different but reproducible constellation of symptoms;
- (6) Concomitant food, alcohol and medication intolerance, estimated to occur in a sizable percentage of MCS patients (Miller, 1994, p. 261).

The olfactory-limbic model for the develop-

ment of MCS (Bell et al., 1992; Bell, 1994) proposed that a combination of the processes involved in kindling and kindling-like phenomena, especially those processes that relate specifically to partial kindling (Adamec and Stark-Adamec, 1983) and the processes that mediate TDS (Antelman, 1988), can account for the development and persistence of the symptoms associated with MCS. Except for the face validity associated with the similarities between sensitization induced by kindling or TDS and that sensitization presumed to accompany the development of MCS, a formal hypothesis has never been proposed to explain specifically how kindling-related phenomena are involved in MCS.

Independent from the context of MCS, the kindling effect has been suggested as a model for sensitization (Martinez-Selva, 1987). As was previously described, electrical kindling shares some substrates with those which underlie simpler sensitization phenomena such as LTP and PTP. Additionally, the transfer of sensitization between electrical kindling and chemical kindling (Kilbey et al., 1979; Cain, 1980, 1982, 1987), and their correspondence after pharmacological manipulations (e.g. Pinel and van Oot, 1975), suggest the existence of similar common substrates for the two phenomena. Clearly, the capability of exhibiting sensitization appears to be a common and unique attribute of nervous system tissue. Unfortunately, the ambiguities surrounding the nature of the relationships between the underlying mechanisms of kindling and related neural sensitization phenomena make it nearly impossible to speculate about which specific underlying mechanisms might be involved in the development of MCS.

As a model for MCS, kindling and kindling-related phenomena share the same problems as they do as a model for short- and long-term memory. For example, Larrabee and Bronk (1947) presumed that learning might be explained by a long persistence effect of nervous impulses caused by a mechanism related to PTP. Bliss and Lomo (1973), in the study of LTP in the perforant path input to the hippocampus, suggested that their findings were significant because LTP would provide a mechanism for hippocam-

pal neurons to retain information for sufficient duration to be potentially useful for information storage. While it is clear that neither PTP nor LTP offer the sensitization permanence necessary to account for long-term memory, their involvement in the processes that lead to the encoding of the engram can not be discounted. It is possibly through their involvement in the processes that subserve kindling that the sensitization induced by PTP and LTP is involved in learning (Goddard and Douglas, 1975; Racine et al., 1975b; Racine and Zaide, 1978; Cain, 1989b). If the mechanisms that underlie PTP and LTP play a role in the development of MCS, their roles are probably similar to those proposed for their participation in learning and memory.

From the initial description of kindling by Goddard (1967), through his work with the kindling phenomenon that proceeded into the 1980s, his fascination with the idea that kindling produced the characteristics necessary to serve as a model for the formation of the long-term memory engram remained apparent. The idea that kindling might represent the engram formation process was suggested by the facts that, like learning, kindling: (a) involves non-degenerative change in neural processing based on relatively permanent alterations of excitatory synapses; (b) can be induced by specific neural activation at specific locations in the mammalian brain; (c) affects behavior in a lasting way; and (d) exhibits the properties of transfer, interference and spontaneous recovery (Goddard and Douglas, 1975). In order to illustrate their position that kindling may still model the process of learning, even though it is an epileptogenic phenomenon induced by electrical currents far in excess of those with any physiological meaning, the authors stated: "a bizarre parent may have a bizarre child through an entirely normal act of procreation" (Goddard and Douglas, 1975, p. 386). One of the few instances of non-inferential support for this hypothesis is provided by the finding that both kindling and learning share the common property of a molecular mechanism which involves calcium-dependent protease associated with postsynaptic membranes, and the activity of this mechanism results in the unmasking of excitatory

glutamate receptors on the membranes, which could partially account for both phenomena (Baudry, 1986).

Both PTP and LTP require high frequency, high amplitude stimuli, and all forms of kindling require the elicitation of either focal afterdischarge or generalized paroxysmal activity in order to achieve the target endpoint. If such corresponding abnormal EEG activity were routinely detected in MCS patients, a physiological potentiation or kindling explanation for the phenomenon could be easily rendered. As is the case for learning, however, neither the sensitization nor triggering phases of MCS is associated with any detectable epileptic paroxysms, nor do individuals afflicted with MCS exhibit any unusual tendency for epilepsy (Bell et al., 1992). In further comparison of models of learning and MCS based on kindling and kindling-related phenomena, several important differences become apparent. Whereas the synaptic events that mediate simple learning are reasonably well studied in animals, no such focal animal model exists for MCS. Similarly, while animal learning can be induced by stimulation of specific brain structures, no corresponding work has been performed with chemical sensitivities. Indeed, because of the lack of testable animal models, kindling may represent an even more "bizarre parent" for MCS than it does for learning and memory.

Direct olfactory stimulation represents the most likely route of exposure involved in MCS. While inhalation exposures through the lung obviously occur, the concentration of inhaled chemicals is probably insignificant. Therefore, while blood-borne chemical contaminants may contribute to some systemic chemical kindling-like effect, it is more probable that a localized chemical kindling-like effect, similar to that observed in ICL chemical kindling, is responsible for inducing the sensitization. Accordingly, olfactory pathways and specifically the olfactory bulbs are particularly sensitive to electrical and chemical kindling (Cain, 1977; Sato et al., 1990). The receptors in the olfactory epithelium form a direct access pathway to olfactory structures in the CNS. It is reasonable, therefore, to assume that

strong activation of the olfactory epithelium cells could provide sufficient input to central olfactory circuits to cause sensitization. Such an effect could be easily studied using standard neurophysiological assessments of central olfactory structures, the amygdala and related limbic structures in response to chemical stimulation of the olfactory epithelium. Unfortunately, no such direct test of the olfactory-limbic hypothesis of CNS sensitization has ever been reported.

Acknowledgments

The opinions and assertions contained herein are those of the author and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large. The experiments reported on herein were performed according to the principles set forth in the current edition of the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council. This work was supported by the Office of Naval Research under Naval Medical Research and Development Command work unit 63706NM0096.004-1516. The author acknowledges Dr. Glenn D. Ritchie for his invaluable assistance in editing and assembling this manuscript. Additionally, the work described here would not have been possible without the accomplished efforts of HM1 Michael Buring, HM3 Jay Smith, HM3 Tracy Morton, HM2 John Gregg, HM3 Timothy Moore, Ms. Cynthia Ademujohn, Ms. Mandy Langley, Mr. William Binole and Ms. Lana Martin.

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Toxicology 111 (1996) 101-117

TOXICOLOGY

Clinically relevant EEG studies and psychophysiological findings: possible neural mechanisms for multiple chemical sensitivity¹

Iris R. Bell*

Departments of Psychiatry and Psychology, University of Arizona and Department of Psychiatry, Tucson Veterans Affairs Medical Center, 3601 S. 6th Avenue, Mail Stop 116A, Tucson, AZ 85723, USA

Abstract

This paper addresses the evidence for the face, construct, and criterion-related validity of the olfactory-limbic/neural sensitization model for multiple chemical sensitivity (MCS). MCS is a poorly-understood, controversial condition in which low levels of environmental chemicals are reported to trigger disabling levels of illness in certain individuals. Neural sensitization processes could generate an endogenous amplification of responsivity to exogenous substances, thereby providing a plausible explanation for the apparent lack of a classical toxicological dose-response relationship in MCS. Convergent data from both survey and psychophysiological studies of MCS patients and of persons from the community without MCS, but who report elevated frequency of illness from chemical odors (cacosmics), support the involvement of the limbic system and the sensitizability of cacosmics, as predicted by the model. Recent studies show that cacosmics do sensitize their heart rate, blood pressure, and plasma β -endorphin responses to repeated exposures to a novel laboratory procedure involving dietary manipulations over time. Cacosmia may represent a pathological form of neural plasticity. Taken together, the model and the available evidence suggest the need for more intensive investigation of MCS from the standpoint of possible neurobiological mechanisms affecting cognitive, emotional, and somatic functions.

Keywords: Sensitization; Olfactory; Limbic nervous system; Environmental chemical intolerance; Validity

1. Introduction

This paper summarizes the olfactory-limbic, neural sensitization model for multiple chemical sensitivity (MCS) (Bell et al., 1992; Bell, 1994a) with a focus on relevant recent data, primarily from our laboratory, for the validity of the model. Although there is no generally accepted case definition (Cullen, 1987; Ashford and Miller, 1991; National Research Council, 1992; Nethercott et al., 1993), MCS is a chronic polysympto-

*Corresponding author, Department of Psychiatry, Tucson Veterans Affairs Medical Center, 3601 S. 6th Avenue, Mail Stop 116A, Tucson, AZ 85723, USA. Tel.: 520-792-1450 x6392; Fax: 520-629-4632.

¹Presented in part at the Tri-Service Toxicology, US Environmental Protection Agency, and Agency for Toxic Substances and Disease Registry Interagency Conference on Risk Assessment Issues for Sensitive Human Populations, Wright-Patterson Air Force Base, Ohio, April 25-27, 1995.

matic, multisystem condition characterized by self-reported susceptibility to illness from low levels of multiple environmental chemicals. Central nervous system (CNS) symptoms such as difficulty concentrating, memory problems, fatigue, depression, dizziness, daytime sleepiness, "spaciness" (derealization), tension, and irritability are common (Miller, 1994; Miller and Mitzel, 1995); somatic symptoms such as joint and muscle pains, gastrointestinal distress, weakness, headache, recurrent sinusitis and bronchitis and other inflammatory conditions, breast and ovarian cysts, and nasal allergies are also frequent (Bell et al., 1994, 1995a, 1996; Buchwald and Garrity, 1994). Patients claim that symptoms flare during chemical exposures and remit during avoidance. Various proposals for possible mechanisms for the condition have included, (i) presumptively psychogenic factors such as odor conditioning or misattribution of psychiatric symptoms such as depression, anxiety/panic, or somatization, (ii) immune system dysfunction; (iii) nervous system dysfunction in the periphery and/or CNS (Ashford and Miller, 1991; Kilburn, 1993; Miller, 1994). As it is beyond the scope of this paper to address the alternative models other than neural sensitization in detail; the reader is referred to several recent reviews for additional perspectives (Bell et al., 1992; Meggs, 1993; Bell, 1994a; Sparks et al., 1994). Nonetheless, the fact that only a subset of people from the general population or from any given chemically-exposed cohort develops MCS (Ashford and Miller, 1991; Cone and Sult, 1992; Miller, 1994) highlights that the focus must be on individual differences in susceptibility to environmental factors more than on inherently toxic properties of the triggering agents themselves (Bell, 1994a).

In order to evaluate the face validity of any model, it is essential to consider the specific phenomenology of MCS that requires explanation (Miller, 1994). In the intense controversy and debate over the existence and nature of MCS, it is notable that many observers have limited their considerations to two general points, i.e. (i) the apparent overlap in symptoms between some aspects of MCS and common psychiatric conditions, e.g. depression, panic dis-

order, somatization disorder; and (ii) the tendency of MCS patients to show extensive and extreme avoidant behaviors towards chemicals (Brodsky, 1983; Sparks et al., 1994). However, the clinical and research literatures indicate a far more complex illness process (Randolph, 1978).

For example, patients report that the same symptoms are triggered not only by chemically-unrelated substances in ambient air, but also by very different environmental agents such as ingested foods, food additives, and drugs. Unlike the typical psychiatric patient with depression, anxiety, or somatization disorder, MCS patients have low rates of smoking and of alcohol tolerance, use, or abuse (Miller, 1994; Bell et al., 1995a). Sensitivities to chemicals encountered in many different settings can persist for months to years despite long-term avoidance; sensitivities to foods are more variable and can remit after weeks to months. Some items are tolerated under certain circumstances, e.g. while away from home on a trip, but not at home, or vice versa. Patients cannot always identify a specific odor in an environment in which they feel ill. Despite histories of illness from certain foods, MCS patients report paradoxical cravings for and binges on those same items, often sweets or other carbohydrates (Miller, 1994). Onset of adverse reactions to triggering agents can begin within seconds, especially in the case of inhaled chemicals or be delayed up to 24 h or more, especially in the case of ingested foods. A single reaction can last for hours to days and can include both activated and deactivated states such as irritability followed by profound sleepiness. Women outnumber men with MCS approximately 4 to 1 (Ashford and Miller, 1991; Buchwald and Garrity, 1994).

The initiation of MCS is distinct from subsequent elicitation or triggering. That is, some patients report an identifiable isolated toxic or peak exposure event (Morrow et al., 1991) following which vulnerability to low levels of chemicals developed without any prior history of such a problem. Others cannot pinpoint a specific causative agent or exposure; they report a more gradual decline into poor health. Most studies have found that the average age of onset of

clinical illness in MCS is age 30 or later (Doty et al., 1988; Black et al., 1990; Ashford and Miller, 1991), in contradistinction to the diagnostic criterion of onset before age 30 for somatization disorder (Bell, 1994a,b). At least some cases of MCS do not derive from an iatrogenic belief system. Clinicians have observed that sporadic cases present with MCS-like histories, but deny any prior awareness of information about MCS from doctors, friends, family, books, or other media.

The distinctive symptom of MCS is cacoscopia, i.e. self-reported illness from low levels of chemicals such as perfume or gasoline that are neutral or mildly unpleasant to unaffected individuals (Ryan et al., 1988). We have developed a Cacoscopia Index screening scale, which is the sum of self-rated frequency of illness on a 5-point Likert scale from five common environmental chemicals (pesticide, paint, perfume, car exhaust, and new carpet) (Bell et al., 1994). MCS patients score an average of 22 on this Index (possible range 5–25) (Bell et al., 1995a). However, cacoscopia is not confined to MCS. Bell et al. (1993a,b,c,d,e, 1996) have surveyed nonindustrial samples of several thousand college students and active retired elderly; in those samples, they demonstrated the presence of a self-reported chemically sensitive subset with a prevalence of 10–30% (dependent upon the phrasing of the questions and application of cutoff criteria). Moreover, a substantial proportion of such persons give personal and family health histories that overlap those of MCS patients, even though they deny having made any avoidant lifestyle changes because of chemical sensitivity. In addition, Morrow et al. (1990) at the University of Pittsburgh have reported cacoscopia in approximately 60% of solvent-exposed workers. Wallace et al. (1991) detailed cacoscopia in 30% of office workers at several of the Environmental Protection Agency's buildings in Washington, DC; this finding was consistent across buildings, even though only one had been conspicuously labelled a "sick building" by some of its inhabitants. Recently, Buchwald and Garrity (1994) noted cacoscopia in persons who met criteria for chronic fatigue syndrome and fibromyalgia, two overlapping conditions with their

own associated complexities and controversies. The degree of cacoscopia in the latter disorders was less than in MCS.

2. Summary of the olfactory-limbic and neural sensitization model

This model (Bell et al., 1992; Bell, 1995a) proposes that cacoscopia and many features of MCS are manifestations of time-dependent sensitization of CNS pathways responsible for regulation of attention/memory, affect, and somatic function (Fig. 1). Cacoscopics are persons with inborn or acquired increases in the capacity to sensitize to the environment, especially to chemicals, but also to other classes of environmental factors such as noise. That is, cacoscopia may reflect pathological plasticity of the nervous system.

In brief, this CNS-based model for MCS involves two elements, (i) *neuroanatomical*—involvement of limbic pathways, including but not limited to, amygdala and hippocampus, that receive input from and/or send output to olfactory and other motivational systems in the brain processing sensory and other environmental information, e.g. mesolimbic and hypothalamic regions. For example, the amygdala is a highly sensitizable limbic structure that plays a role in processing olfactory sensory information, generating emotions, modulating social behaviors, autonomic activity, and reproductive endocrine function, and initiating context-dependent sensitization of other behaviors; (ii) *neurobiological*—participation of a time-dependent sensitization (TDS) process in initiation and elicitation of susceptibility to low levels of environmental chemicals.

TDS is the progressive amplification of responses by the passage of time between the repeated, intermittent exposure to a given environmental factor (Antelman, 1988, 1994; Antelman et al., 1979, 1980, 1991, 1992a,b). The nature of the factor can include pharmacological or chemical agents or physical or psychological stressors. Pharmacological and nonpharmacological stimuli can cross-sensitize. Initiation of TDS may depend in part upon participation of

mesolimbic pathways involving the dopaminergic ventral tegmental area (VTA) (Kalivas and Stewart 1991; Kalivas et al., 1993). Excitatory amino acids, which are essential in many different brain processes involving neural plasticity and learning, also participate in initiation of TDS (Snyder-Keller, 1991; Kalivas and Alesdatter, 1993; Pinheiro-Carrera et al., 1995). TDS can be bidirectional; low level initiating stimuli can induce an increase, whereas high level initiating stimuli can induce a decrease or reversal in the subsequent sensitized response to another stimulus (Antelman et al., 1991). A special case of TDS may be limbic kindling, e.g. of olfactory bulb or amygdala, in which daily repeated low level electrical or chemical stimulation that has no initial effect eventually induces permanent susceptibility to tonic-clonic seizures (Joy, 1982; Weiss et al., 1986; Adamec, 1990, 1994; Gilbert, 1992).

Various forms of TDS and/or kindling are models not only for many of the disorders reported at increased rates in MCS, including recurrent depression (Post et al., 1984; Post, 1992), panic disorder (Dager et al., 1987), somat-

ization disorder (Teicher et al., 1993; Bell, 1994b), post-traumatic stress disorder (Yehuda and Antelman, 1993), and chronic pain (Coderre et al., 1990, 1993; Cervero and Janig, 1992; Ursin et al., 1993; Watkins et al., 1994), but also for substance abuse (Post et al., 1976; Post, 1980; Newlin and Thomson, 1991; Sorg and Kalivas, 1991; Hunt and Lands, 1992; Jackson and Nutt, 1993; Kalivas et al., 1993; Robinson and Berridge, 1993), a disorder reported at decreased rates in MCS (Bell et al., 1995a). Current studies suggest that chronic pain may result from sensitization of both peripheral nociceptors and of CNS regions such as dorsal horn in the spinal cord and limbic structures such as the cingulate bundle and fornix (Vacarino and Melzack, 1992; Coderre et al., 1993). The basic science research suggests that cacoscopia and its different clinical manifestations could involve multiple convergent routes to heightened reactivity to low levels of chemicals (Bell et al., 1992): i.e. (i) inborn, genetically-based "hard-wiring" of hyperreactivity in relevant limbic and/or mesolimbic circuits such as trait shyness (see Kagan et al., 1988); (ii) acquired CNS

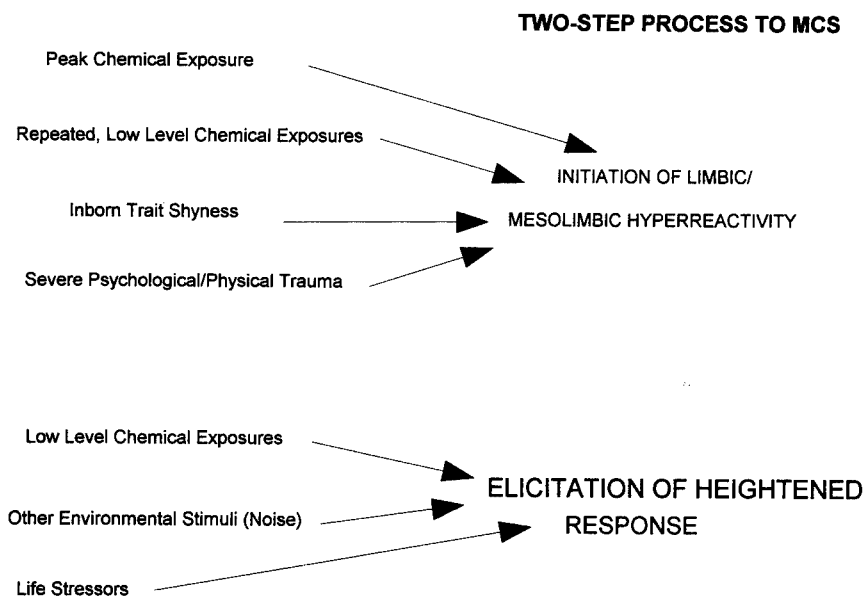


Fig. 1. Model for a two-step process (initiation and elicitation) in the development of multiple chemical sensitivity. Different classes of stimuli act on the same pathways and processes.

hyperreactivity following earlier life psychological or physical trauma such as childhood abuse, natural disasters, accidents, or surgeries (see Teicher et al., 1993); (iii) acquired CNS hyperreactivity following a single peak exposure to a toxic environmental chemical (see Mutti and Franchini, 1987; Mutti et al., 1984; Morrow et al., 1991) causing neural injury with associated excitatory amino acid and dopamine release; (iv) acquired CNS hyperreactivity following repeated, low level exposures to novel stressors and/or environmental chemicals (see Burchfiel and Duffy, 1982); (v) other, as yet undefined pathways. More extensive discussions of the olfactory-limbic and neural sensitization model are available in several recent papers (Bell et al., 1992, 1995g; Antelman, 1994; Bell, 1994a; Bell and Schwartz, 1995). The remainder of this paper will summarize findings related to the model's potential validity.

3. Face validity of the model

Neural sensitization has face validity as a model for MCS in that the essence of both sensitization and MCS is amplification of responses within the individual to an environmental stimulus which by itself at first cannot evoke such large responses. As such, it answers the greatest concern of some classical toxicologists, who, finding no apparent dose-response relationship in MCS (low doses eliciting big responses), dismiss it as biologically impossible. The model also accommodates key features of the clinical phenomenology, especially the striking involvement of the CNS in the leading symptoms (Black et al., 1990; Simon et al., 1990, 1993; Cone and Sult, 1992; Miller, 1994). It could explain past observations in MCS patients, such as the increased rates of certain comorbid psychiatric disorders (Black et al., 1990; Simon et al., 1990, 1993), without necessarily reducing MCS to being a psychogenic disorder (Bell, 1994).

Furthermore, neural sensitization can interface plausibly with other leading models for MCS, including classical conditioning (Bolla-Wilson et al., 1988) or neurogenic inflammation (Meggs, 1993). That is, TDS can occur in either context-

dependent (conditioned) or context-independent (unconditioned) fashion, depending upon the design of the original sensitizing and subsequent testing procedures (Post et al., 1984; Vezina and Stewart, 1990; Stewart and Vezina, 1991). At the same time, sensitization to the unconditioned stimulus is a long-recognized nonassociative learning feature of classical conditioning, apart from the associative learning involved in acquiring responses to a conditioned stimulus (Thompson and Spencer, 1966; Groves and Thompson, 1970). In addition, nonimmunologic, neurogenic inflammation in peripheral tissues has been postulated as a mechanism for some of the somatic conditions associated with MCS (Meggs, 1993). Using parallels drawn from research on chronic pain, evidence suggests that lesions of certain limbic structures prior to experimental peripheral injury from formalin can prevent development of later pathological pain (Vaccarino and Melzack, 1992). Preventing input from peripheral nociceptors to the CNS before but not after injury also attenuates the development of later pathological pain (Coddere et al., 1993). In other words, inflammatory processes in the periphery may send sensitizing input to the CNS, which in turn develops and perpetuates a chronic hyperreactivity, later independent of any persistence of peripheral injury (Cervero and Janig, 1992; Coderre et al., 1993). Such a hypothesis has been proposed for irritable bowel (Cervero and Janig, 1992), one of the common diagnoses claimed by MCS patients and community cacosmics (Bell et al., 1994, 1995a).

4. Construct validity of the model

4.1. *Limbic and related pathway involvement*

Convergent lines of evidence are consistent with the neural sensitization model for MCS and cacosmia. If cacosmics have hyperreactive limbic systems (Bell et al., 1995g), then they should score higher on questionnaires assessing limbic dysfunction. Fig. 2 illustrates that college students in the top quartile for cacosmia scores score significantly higher than all of the other quartiles on the McLean Limbic Symptom Checklist (Teicher et al., 1993), a 33-item scale

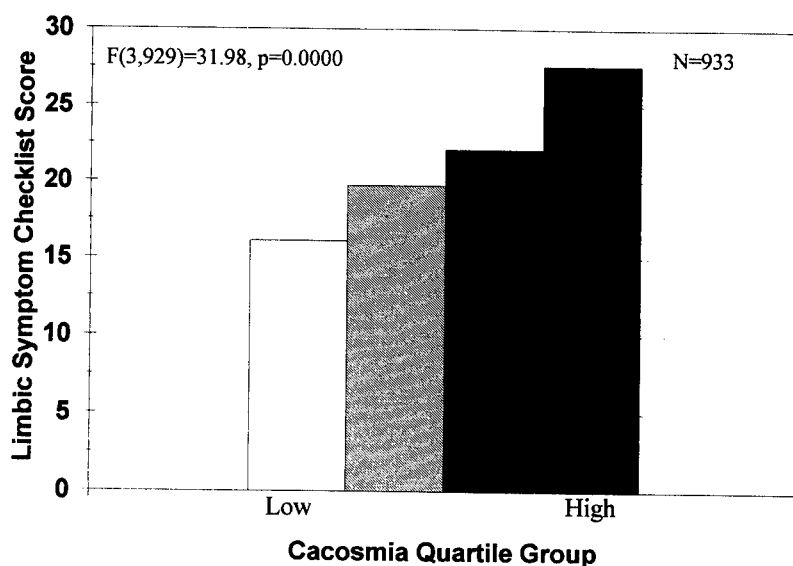


Fig. 2. Higher scores on the limbic symptom checklist are associated with higher cacostmia ratings in young adult college students (highest quartile > all other quartiles, $P < 0.05$ post hoc test; lowest quartile < all other quartiles, $P < 0.05$ post-hoc test).

involving the ictal sensory, behavioral, memory, and somatic features of temporal lobe epilepsy (the amygdala contains the seizure focus in a large proportion of cases). MCS patients and other cacostmics both report heightened startle reactions (Bell et al., 1996). The amygdala plays a major role in modulating the startle response (Ornitz and Guthrie, 1989). As a result, cacostmics should score higher than noncacostmics on measures of subjective noise sensitivity; and they do (Bell et al., 1995g) (see Fig. 3). Women with temporal lobe epilepsy have increased rates of dysfunction in their reproductive system, including elevated prevalence of polycystic ovary disease (Herzog et al., 1984). MCS patients also have increased rates of physician-diagnosed ovarian cyst histories (Bell et al., 1995a). Herzog et al. (1984) have proposed that a dysfunctional amygdala in the epileptic women disrupts the normal regulation of hormonal output from the hypothalamus, thereby setting up the anovulatory, unopposed estrogen cycles that favor cyst formation at the ovary. Notably, unopposed estrogen may also contribute to breast cyst formation, another common symptom for both MCS pa-

tients and other cacostmic women (Bell et al., 1994, 1995a, 1996).

Theta activity (Sainsbury et al., 1987; Schwartz et al., 1994), a slower electroencephalographic (EEG) frequency (e.g. 4-8 Hz), is considered by some to be an objective correlate of the subjective symptom of "spaciness," i.e. derealization or depersonalization (Mann et al., 1992) and of poorer performance on vigilance tasks (Beatty et al., 1974; Valentino et al., 1993). Derealization is a frequent symptom not only in temporal lobe epileptics, but also in MCS and other cacostmics. Staudenmayer and Selner (1990) reported increased resting theta power over the parietal region of the brain in MCS patients and mixed psychiatric patients, compared with normal controls. We have found elevated resting theta activity over the same temporoparietal region after a number of different tasks in cacostmic young adults who were not depressed (Bell et al., 1995c). While Staudenmayer et al. (1993) reported increased rates of childhood sexual abuse histories in MCS patients, Teicher's group have shown increased scores on the McLean Limbic Symptom scale for adult psychiatric patients with

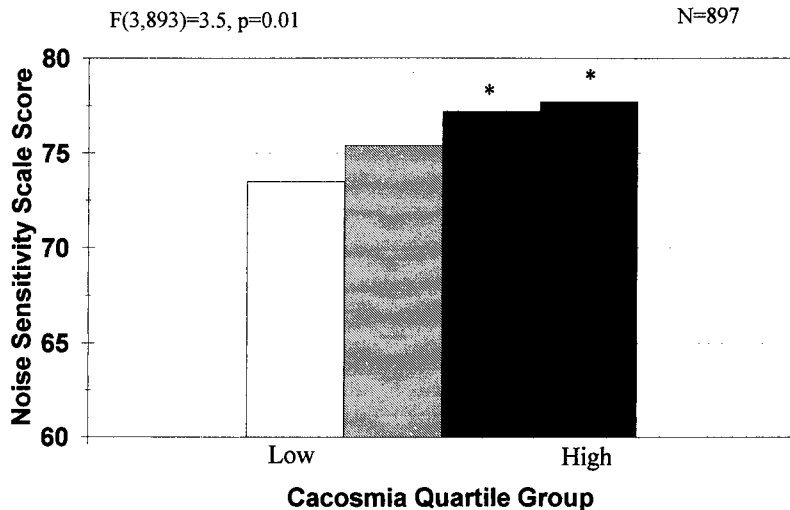


Fig. 3. Higher scores on the noise sensitivity scale are associated with higher cacostmia ratings in young adult college students ($P < 0.05$ post-hoc test, two highest quartiles $>$ lowest quartile).

histories of all types of childhood abuse compared with psychiatric patients without such histories (Teicher et al., 1993). Moreover, Ito et al. (1993) demonstrated that children with childhood abuse histories (but no head trauma) have increased rates of electrophysiological abnormalities over the frontal, temporal, or anterior regions, especially on the left side. These abnormalities could reflect a cause or a result of the abuse, or an inherited trait; but, regardless of how or when brain dysfunction developed, its presence in abused persons indicates a current potential vulnerability to abnormal reactivity to environmental stimuli that affect the limbic system, which would include olfactory stimuli, foods, and drugs, as well as life stressors (Bokina et al., 1976; Kalivas et al., 1986; Kavaliers and Innes, 1988; Konsarska et al., 1989; Mitchell and Gratton, 1992a,b; Mokrushin and Emelyanov, 1993).

Problems with concentration, attention, and memory are among the most disabling and most frequently reported symptoms in MCS patients (Bell et al., 1995c; Miller and Mitzel, 1995). The electrode sites at which the strongest findings in MCS and other cacostmics have been seen are in the temporoparietal region, e.g. T5, P3, Pz, P4,

T6 in the 10–20 international system for EEG (Staudenmayer and Selner, 1990; Bell et al., 1995c). Of note, the parietal cortex is involved in the processing of complex aspects of attention (Mesulam, 1985) as well as of olfactory stimuli (Kobal et al., 1995). This overall region, especially temporal, also participates in formation of memory in conjunction with the hippocampus. Emerging data from Fiedler et al. (1992, 1994) suggests that MCS patients may have their greatest cognitive difficulty in detecting target information from background noise, perhaps especially in visuospatial rather than verbal arenas. This attentional dysfunction may influence ability to perform on certain aspects of learning and memory tasks. These considerations would parallel the suggestion of Morrow et al. (1990; see also 1989, 1992) from the visuospatial deficits in neuropsychological test performance of solvent-exposed workers that right parietal cortex may be particularly susceptible to solvents. Consistent with these considerations, we found that elderly cacostmics had significantly slower reaction times but normal tracking times on a complex divided attention task in the laboratory (Bell et al., 1995d). In other words, as others have found in MCS patients (Simon et al., 1993), we

observed that cacosmics can follow a single stimulus to which they must attend, within normal capacity. However, when overloaded with additional competing attentional demands on a visual attention task, older cacosmics cannot perform as well as normals on the added aspects of the test. Greater Cacosmia Index score also correlated with higher scores on the self-rated Confusion subscale (subjective cognitive inefficiency) of the Profile of Mood States Scale in the latter study (Bell et al., 1995e,f). Whether or not these findings will generalize to MCS patients and younger cacosmics is also now under study.

Insomnia at night and sleepiness during the day are common symptoms in MCS patients and other cacosmics. In sleep research, however, it is well known that many people complain of difficulty sleeping but demonstrate objectively normal sleep on polysomnographic recording. As earlier research has often failed to find objective support for various other symptoms and concepts in MCS, it is important to test sleep directly. We found that elderly cacosmics had objectively lower total sleep time than did their noncacosmic peers (Bell et al., 1995f). In contrast with the sleep structure of persons with depression, these cacosmics had a trend toward a later onset of their first rapid-eye-movement sleep period (REM) and lower total REM as a percent of sleep period. Even after forcing various psychological variables first into a regression equation, Cacosmia Index still accounted for a significant portion of the total sleep time and REM %. Although several neurochemical scenarios could underlie this sleep pattern, one possibility is an increase in dopaminergic function in certain brain areas, which would be a potential correlate of a sensitized mesolimbic system (Kalivas et al., 1993; see also Callaway et al., 1994). Amygdala kindling can similarly alter sleep patterns (Stone and Gold, 1988). Psychopharmacological studies could assist in evaluating specific alternative hypotheses for the sleep findings.

4.2. *Time-dependent sensitization processes*

If cacosmics are highly sensitizable, then they should have the individual differences that basic researchers have found to characterize highly

sensitizable animals: (i) genotypic vulnerability (Cabib and Puglisi-Allegra, 1991; Kosten et al., 1994); (ii) female gender (Robinson et al., 1982; Robinson and Becker, 1986); (iii) increased glucocorticoids (Deroche et al., 1992, 1993, 1994); (iv) hyperreactivity to novelty (Hooks et al., 1992); (v) increased spontaneous preference for sucrose (Sills and Vaccarino, 1993); (vi) differential brain lateralization (LaHoste et al., 1988) (e.g. left-handedness). In the case of (i) genotype, we reasoned that family health histories might provide a surrogate for genetics. The most well-recognized disorder in which TDS is believed to play a role is craving in drug abuse (Robinson and Berridge, 1993). Thus, we predicted that cacosmics should inherit sensitizability and thus have higher rates of family substance abuse problems, even though they themselves do not tolerate drugs. The reasoning would be that cacosmics are so sensitizable, they develop the aversive rather than rewarding effects of many substances when they sensitize. The result is avoidance rather than approach to many substances; however, they should retain the ability to crave some items with more positive and less immediately negative effects, i.e. certain foods. As predicted, Fig. 4 demonstrates that the upper two quartiles of a college student sample for cacosmia report higher rates of physician-diagnosed drug problems in blood relatives than do the bottom two quartiles (see also carbohydrate craving data below).

Female animals sensitize to drugs and stress better than do males, perhaps due to a permissive effect of estrogens (Peris et al., 1991). In the case of gender differences, various clinical researchers have reported that women far outnumber men among MCS patients (Ashford and Miller, 1991). Although it is possible that this gender differential derives artifactually from the fact that women see doctors and report health problems more readily than do men, our surveys on college students and active retired elderly from the community suggest that the gender difference for cacosmia also holds in the non-clinical, nonindustrial population. Women constitute approximately 70-80% of most cacosmic groups in those studies, whereas the gender distribution is more

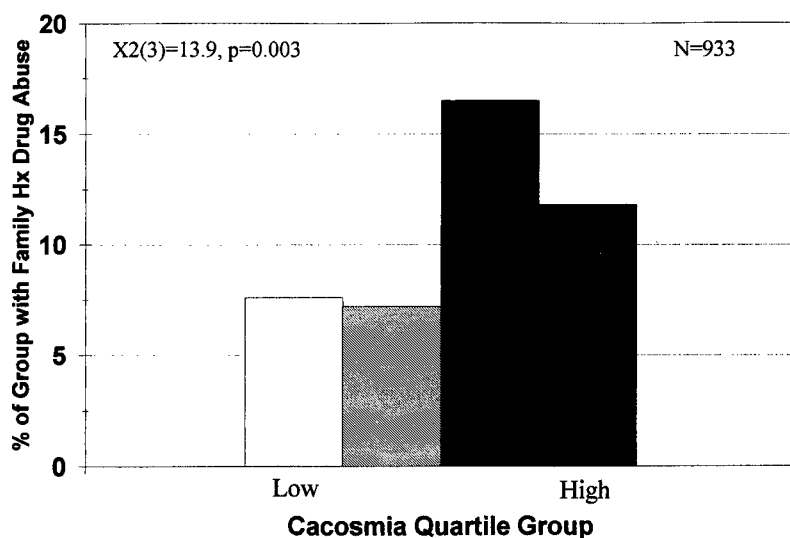


Fig. 4. Increased prevalence of familial drug abuse histories in young adult college students with higher cacoscopia ratings (third quartile > lowest two quartiles, $P < 0.05$ post-hoc test).

equal at 50% in the least cacoscopic groups (Bell et al., 1993c,d, 1996).

Cortisol and related hormones may be key participants in sensitization. Adrenalectomy can prevent the induction of sensitization in animal studies (Deroche et al., 1992). Higher initial levels of corticosterone in animals predict drug sensitization. Intact animals sensitized to a drug then exhibit stronger glucocorticoid output to a novel stressor than do non-sensitized animals. If cacoscics are more sensitizable and/or more sensitized, they should exhibit greater cortisol responses to environmental novelty. We studied the 0900 h blood levels of β -endorphin in elderly cacoscics after the novel stress of sleeping all night in a university sleep research laboratory and undergoing cognitive testing and milk or soy-based beverage ingestion in the morning. Beta-endorphin is the endogenous opioid peptide hormone released during activation of the hypothalamic-pituitary-adrenal axis with ACTH. As predicted, averaged over six mornings spread over a 6-week period, the cacoscics had significantly higher levels of plasma β -endorphin than did the noncacoscopic controls (Bell et al., 1995e). Thus, as predicted by a sensitization model, un-

der the stress of a novel laboratory experience, cacoscics show evidence of heightened activation of the physiological stress response system. We are currently examining cortisol output directly in another study.

One of the most reliable behavioral indicators of elevated sensitizability is hyperreactivity to novelty. Animals who show spontaneously high rates of activity when placed in a novel physical environment later sensitize more strongly to drugs or stress (Hooks et al., 1992). A potential human parallel for one extreme of novelty hyperreactivity is trait shyness. Kagan et al. (1988) have demonstrated that children with inborn shyness have increased salivary cortisol output at home and in the laboratory, as well as increased nasal allergies; they react to the unfamiliar or novel with tension, fearfulness, and withdrawal. Shy or inhibited babies cry and kick when confronted with a new mobile over their crib, whereas outgoing babies show pleasure at the stimulus. An animal model for shyness is partial kindling of the amygdala (Adamec, 1990). Thus, assuming both limbic involvement and sensitizability in cacoscics, they should be more shy than non-cacoscics. As predicted, cacoscics have shown

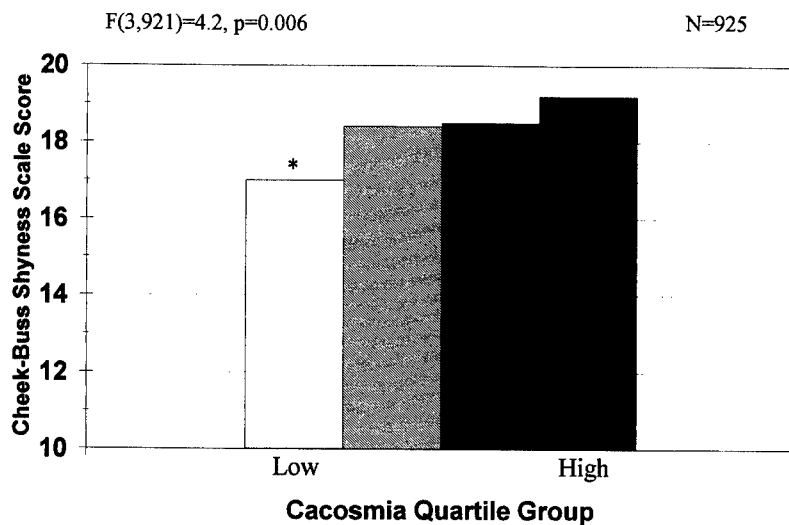


Fig. 5. Lowest cheek buss shyness scale scores in young adult college students lowest in cacoshmia ratings (* $P < 0.05$ post-hoc test, lowest quartile < all other quartiles).

increased shyness on standardized scales in most, though not all, of our studies (Bell et al., 1993a,c,d, 1994, 1995g, 1996) (Fig. 5).

Recent investigations showed that animals that spontaneously ingested more sucrose than their peers later exhibited greater sensitizability to

drugs (Sills and Vaccarino, 1993). Thus, the human analogy should be increased craving for sweets. In our last college student survey (Bell et al., 1995g), we administered the Carbohydrate Addict's Test, a self-report scale of carbohydrate preference and craving. As predicted by the TDS

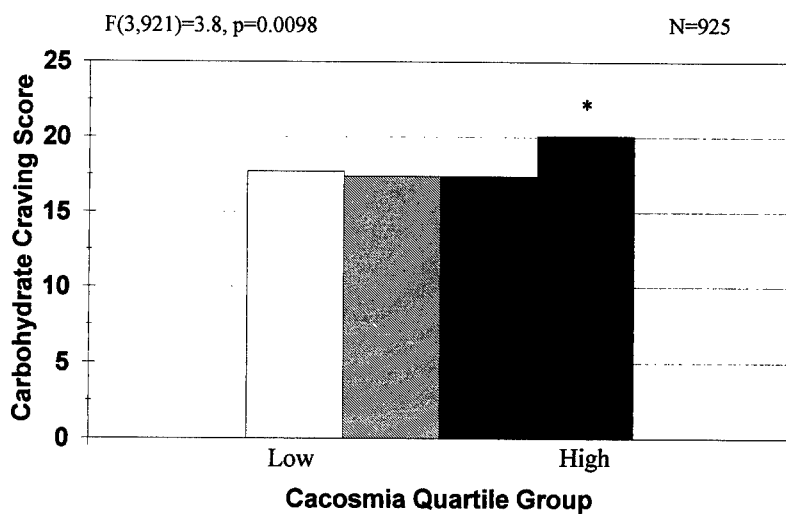


Fig. 6. Highest carbohydrate craving scales scores in young adult college students with highest cacoshmia ratings (* $P < 0.05$ post-hoc test, highest quartile > all other quartiles).

model, the most cacosmic quartile scored the highest for carbohydrate "addiction" (Fig. 6).

Finally, one study of brain lateralization in animals demonstrated that the subset of animals that showed a strong, consistent turning leftward after a test dose of stimulant drug later sensitized the most to the drug. In a previous college student survey, we did not find any difference in the prevalence of left-handedness among cacosmics chosen on the basis of their answers to questions about frequency of illness from chemical odors. However, we did find greater lefthandedness in the approximately 30% of the sample who endorsed affirmatively a single question about whether or not they "consider [themselves] especially sensitive to certain chemicals" as compared with those who denied a self-perception as a chemically-sensitive person (13% vs. 7%) (Bell et al., 1996).

5. Criterion-related validity of the model

5.1. Predictive validity

If cacosmia is a manifestation of time-dependent sensitization, then cacosmics should show sensitization of various physiological and/or psychological/behavioral measures to various environmental factors, including but not limited to chemicals. We have demonstrated sensitization of cardiovascular measures (Bell et al., 1995b) and of plasma β -endorphin levels (Bell et al., 1995e) in cacosmic elderly individuals to a novel laboratory situation, including food ingestion. In our sleep laboratory study, subjects slept in the laboratory over three pairs of nights while on baseline (last two days of a 2-week period), dairy-containing (last two days of a 3-week period), and non-dairy (last two days of a 3-week period) diets. Baseline diet (with ad lib milk) was always first; the order of the subsequent dairy and non-dairy diets was counterbalanced within groups. We measured heart rate and blood pressure while subjects were supine upon awakening each laboratory morning. As predicted by the TDS model, we found that cacosmics did not differ from noncacosmics on the first baseline morning, but then showed significantly greater heart rates

on both experimental diets over the next 6-week course of the study. Cacosmics also exhibited higher diastolic blood pressures on the second rather than the first day of the pairs of days across all diets. Similarly, Morrow and Steinhauer (1995) recently reported that solvent-exposed workers fail to habituate their autonomic nervous system responses (heart rate and pupil responses) to the stress of a difficult cognitive task during a single-session study. This habituation failure worsened with the passage of many months on re-testing in the same laboratory in some individuals.

The plasma β -endorphin levels showed a more complex pattern of possible sensitization and habituation. They were highest on Baseline Day 1 but lowered to the range of the noncacosmics' levels on Baseline Day 2 (24 h later). After 3 weeks had passed between laboratory sessions, however, the cacosmics again exhibited a rise in β -endorphin levels, regardless of which diet they had consumed. On the dairy diet, to which they had been exposed at baseline, cacosmics continued to show a rise in endorphin on Laboratory Day 2. In contrast, on soy, to which they had not been exposed at baseline, cacosmics exhibited another drop in endorphin on Laboratory Day 2 of that diet. Taken together, the data suggest that cacosmics have labile plasma β -endorphin status that may fluctuate based on past experience with a given environmental context and even a given food (see Antelman et al., 1991, 1995). Overall, cacosmics show the capacity to sensitize in autonomic and hormonal responses to stimuli other than chemicals. Direct tests of their chemical sensitizability are now underway in our laboratory.

5.2. Correlational validity

Finally, it is important to point out that the Cacosmia Index per se appears to measure a construct that has both overlapping and distinctive properties from those of other measurement scales. For example, Simon et al. (1990) developed a 4-item true/false survey of lifestyle changes associated with chemical sensitivity. They and others have found that MCS patients score an average of 3 of 4 on this scale; we have found that

Table 1
Summary of validity evidence for limbic/neural sensitization model

Face validity

Individual differences in sensitization could account for apparent lack of dose-response relationship (low doses trigger big responses)

Construct validity

Cacosmics have higher scores on Limbic Symptom Checklist

Cacosmic women have more ovarian and breast cysts (see TLE)

Cacosmic elderly perform more poorly on divided attention task (see attention symptom)

Cacosmic elderly have lower objective total sleep times (see insomnia symptom)

Cacosmic young adults have increased theta activity at rest (see "spaciness" symptom)

Cacosmics have sensitizability characteristics: female gender, FH drug abuse, carbohydrate craving, shyness

Cacosmic elderly have increased plasma β -endorphin levels (see HPA activation)

Predictive validity

Cacosmic elderly exhibit sensitization of heart rate and diastolic blood pressure

Cacosmic elderly exhibit sensitization of β -endorphin levels

TLE, temporal lobe epilepsy.

FH, family history.

HPA, hypothalamic-pituitary-adrenal axis.

our community cacosmics regularly score a mean of less than 1 of 4 (Bell et al., 1994). In MCS patients, we found that the Cacosmia Index score correlated well with the Simon Survey ($r = 0.57$, $n = 25$, $P = 0.003$). However, within a previous study of community elderly with few lifestyle changes, Cacosmia Index score correlated moderately with the Simon Survey ($r = 0.39$, $n = 235$, $P = 0.000$). In the elderly, Cacosmia Index had weak but significant correlations with the depression ($r = 0.17$, $n = 185$, $P = 0.02$) and anxiety ($r = 0.19$, $n = 188$, $P = 0.007$) subscales of a standardized measure of psychological distress, the Symptom Checklist 90 (revised) (SCL-90-R). In a different survey, this time in young adults, Cacosmia Index showed similarly low correlations with depression (Beck Depression Inventory: $r = 0.09$, $n = 900$, $P = 0.009$) and anxiety (Beck Anxiety Inventory: $r = 0.15$, $n = 861$, $P = 0.000$), but correlated moderately with measures of somatic concerns, i.e. the SCL-90-R somatization subscale ($r = 0.29$, $n = 928$, $P = 0.000$) and of amplification of somatic symptoms, i.e. the Barsky Amplification Scale ($r = 0.28$, $n = 929$, $P = 0.000$). In these college students, current Cacosmia Index ratings were

highly correlated with ratings for the same items requested for "most of your life" ($r = 0.91$, $n = 933$, $P = 0.000$).

6. Conclusions

Overall, the olfactory-limbic/neural sensitization model offers a testable approach to a controversial and complex clinical problem (Bell et al., 1992; Bell, 1994a; Bell and Schwartz, 1995). Table 1 summarizes the arguments for the validity of the model. At the same time, much of our evidence for the validity of the model derives from studies on non-MCS cacosmics without industrial chemical exposures. It is plausible that this type of cacosmic differs from MCS patients in important ways, most obviously in terms of lesser illness severity and lesser disability. It will be essential in future research to extend these investigations directly to the clinical populations that stimulated the original debate. Nonetheless, cacosmia appears to be a trait along a continuum of frequency and severity within the general population. The factors that turn one cacosmic into an MCS patient and allow another to live with minimal impairment require careful study.

The neural sensitization model can accommodate much of the phenomenology in MCS and other cacosomes. It permits hypothesis-driven research, and it encourages development of animal models as well as of systematic human protocols for both laboratory and field studies in home, office, and factory over time.

Acknowledgements

Supported in part by grants from the National Dairy Board administered in cooperation with the National Dairy Council, BRSG grant (University of Arizona) 2S07 RR05675-23, the Environmental Health Foundation, and the Wallace Genetic Foundation.

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ELSEVIER

Toxicology 111 (1996) 119–134

TOXICOLOGY

Potential animal model of multiple chemical sensitivity with cholinergic supersensitivity

David H. Overstreet^{*a}, Claudia S. Miller^b, David S. Janowsky^a, Roger W. Russell^c

^aCenter for Alcohol Studies and Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7178, USA

^bDepartment of Family Practice, University of Texas Health Science Center, San Antonio, TX 78284, USA

^cCenter for Neurobiology of Learning and Memory, University of California at Irvine, Irvine, CA 92717, USA

Abstract

Multiple Chemical Sensitivity (MCS) is a clinical phenomenon in which individuals, after acute or intermittent exposure to one or more chemicals, commonly organophosphate pesticides (OPs), become overly sensitive to a wide variety of chemically-unrelated compounds, which can include ethanol, caffeine and other psychotropic drugs. The Flinders Sensitive Line (FSL) rats were selectively bred to be more sensitive to the OP diisopropylfluorophosphate (DFP) compared to their control counterparts, the Flinders Resistant Line (FRL) rats. The present paper will summarize evidence which indicates that the FSL rats exhibit certain similarities to individuals with MCS. In addition to their greater sensitivity to DFP, the FSL rats are more sensitive to nicotine and the muscarinic agonists arecoline and oxotremorine, suggesting that the number of cholinergic receptors may be increased, a conclusion now supported by biochemical evidence. The FSL rats have also been found to exhibit enhanced responses to a variety of other drugs, including the serotonin agonists *m*-chlorophenylpiperazine and 8-OH-DPAT, the dopamine antagonist raclopride, the benzodiazepine diazepam, and ethanol. MCS patients report enhanced responses to many of these drugs, indicating some parallels between FSL rats and MCS patients. The FSL rats also exhibit reduced activity and appetite and increased REM sleep relative to their FRL controls. Because these behavioral features and the enhanced cholinergic responses are also observed in human depressives, the FSL rats have been proposed as a genetic animal model of depression. It has also been reported that MCS patients have a greater incidence of depression, both before and after onset of their chemical sensitivities, so cholinergic supersensitivity may be a state predisposing individuals to depressive disorders and/or MCS. Further exploration of the commonalties and differences between MCS patients, human depressives, and FSL rats will help to elucidate the mechanisms underlying MCS and could lead to diagnostic approaches and treatments beneficial to MCS patients.

Keywords: Animal model of MCS; Organophosphate DFP; FSL rats; Human depressives; Cholinergic supersensitivity

^{*}Corresponding author, Skipper Bowles Center for Alcohol Studies, Thurston-Bowles Bldg., CB 7178, University of North Carolina, Chapel Hill, NC 27599-7178, USA. Tel.: (919) 966-5678, Fax: (919) 966-5679.

1. Introduction

Multiple Chemical Sensitivity (MCS) is a clinical phenomenon in which individuals, after acute or intermittent exposures to one or more chemicals, commonly organophosphate pesticides (OPs), report that they become overly sensitive to a wide variety of chemically-unrelated compounds, which can include ethanol, caffeine and other psychotropic drugs (Cullen, 1987; Ashford and Miller, 1989, 1991; Bell et al., 1992; Miller, 1994). Symptoms commonly reported by these individuals include fatigue, cognitive difficulties, depression, irritability, headaches, dyspnea, digestive problems, musculoskeletal pain, and numbness in their extremities. Symptoms often overlap those of better recognized medical illnesses such as depression, somatization disorder, chronic fatigue syndrome, fibromyalgia, asthma and others. However, a distinguishing feature of MCS is the patients' assertion that their symptoms are triggered by common exposures to low levels of volatile organic chemicals (for example, fragrances, insecticides, traffic exhaust, disinfectants), as well as by many different foods, drugs, ethanol and caffeine.

There has been considerable controversy over the existence of this phenomenon, punctuated by the many Gulf War veterans who have complained of similar symptoms and chemical intolerances since returning from the Gulf. Although there now is widespread recognition of MCS as a clinical phenomenon, there is still much uncertainty as to whether it is a definable syndrome, what its etiology is, and what biopsychosocial mechanisms might underlie it.

Relevant information about conditions like MCS is derived from three general sources, all of which supplement one another and each of which has its own limitations. Epidemiological studies often cannot differentiate which among several possible variables is (are) involved in the adverse effects observed. Once a toxic exposure has occurred, clinical research provides valuable information concerning diagnosis, therapy and rehabilitation. However, both epidemiology and clinical studies involve post hoc analyses, i.e. the human exposure has already occurred. A third

source of information in biomedical research — use of animal models—is the only approach which allows experimental manipulations and affords the precision of laboratory measurement. The major concern in using this approach is the validity of extrapolating results to human situations. Empirical tests of this validity have earned animal models an essential role in understanding human toxicities (Russell, 1991; MacPhail and Peele, 1992). Our present objective is to propose that use of a particular animal model will be useful in the search for the etiology of and mechanisms underlying MCS.

The validity of an animal model rests in part on its similarity in structure and function to the human situation. The closer the similarity, the greater is the probability that manipulations of one will provide information valid for extrapolation to the other. A final test of validity comes when predictions are shown to be accurate. To evaluate the model proposed below, it is important to summarize the observed clinical characteristics of MCS.

2. Multiple chemical sensitivity

Anecdotal descriptions of MCS have appeared in the medical literature for more than 40 years. In the past five years, occupational medicine physicians in universities have reported seeing increasing numbers of individuals with this affliction, and three federally-sponsored workshops on MCS have been held (Association of Occupational and Environmental Clinics, 1992; National Research Council, 1992; Mitchell and Price, 1994). Sponsoring agencies have included The National Research Council (NRC), the Agency for Toxic Substances and Disease Registry (ATSDR), the Environmental Protection Agency, and the National Institute of Environmental Health Sciences (NIEHS). Recommendations from these meetings have underscored the need for further research on the condition and the development of animal models.

MCS has been described as a two-step process that in some ways parallels allergic diseases (Ashford and Miller, 1991): (1) *Induction* (initiation,

sensitization or loss of tolerance) as a consequence of an initial chemical exposure (analogous to sensitization to bee venom), and (2) subsequent *triggering* of symptoms by a wide range of chemically-diverse substances (here the analogy to allergy breaks down, since antibodies are highly specific and spreading of sensitivities to chemically unrelated substances does not occur with allergy).

Among the substances MCS patients most frequently report as having induced their condition are pesticides, especially OPs and carbamates (Ashford and Miller, 1991; Miller and Mitzel, 1995). Interestingly, many Gulf War veterans also now report symptoms reminiscent of MCS. Exposures to OP and carbamate agents during the Gulf War included pesticides, the nerve agent pretreatment drug pyridostigmine bromide and, possibly, low levels of nerve agents. Although chemicals in this class can inhibit cholinesterase, rarely are cholinesterase levels measured in sporadic cases, and frequently acute symptoms associated with cholinesterase inhibition are absent among individuals who report developing MCS as a consequence of OP exposure. While OP toxicity has been considered largely reversible if it is not fatal, the toxicology literature contains numerous examples of individuals exposed to these agents who showed persistent neuropsychological deficits (Rowntree et al., 1950; Gershon and Shaw, 1961; Tabershaw and Cooper, 1966; Savage et al., 1988; Rosenstock et al., 1990). Some authors have proposed that OPs may damage cholinergic receptors or in other ways induce injury independent of their ability to inhibit cholinesterase (Gupta and Abou-Donia, 1994; Huff et al., 1994).

With respect to MCS, Rosenthal and Cameron (1991) described a retired attorney with depression whose home was sprayed with an OP pesticide. Subsequently, the attorney's depression became markedly more severe, and he developed multi-system symptoms and new intolerances to odors. These authors hypothesized that cholinergic sensitivity might underlie both environmental sensitivities and depressive tendencies. Cone and Sult (1992) described a group of casino workers exposed to a mixture of carbamate and

pyrethrin insecticides who subsequently developed chronic, multi-system symptoms, cognitive difficulties and sensitivity to the odor of pesticides, perfumes, gasoline, newsprint, and cleaning agents. Soon after their exposure, a number of these individuals, who were dealers, experienced difficulty counting cards. Later they reported feeling ill around tobacco smoke, an exposure they previously had tolerated while at work.

More recently, Miller and Mitzel (1995) surveyed 112 MCS patients, 37 of whom attributed their illness to exposure to an OP or carbamate pesticide and the other 75 to remodelling of a building. Remodelling commonly involves exposures to low levels of mixed solvents emanating from fresh paint, carpeting, glues, etc. Following their initial exposure, both groups reported similar symptoms and similar intolerances to chemicals, foods, ethanol, and caffeine. However, overall, the pesticide-exposed group reported significantly greater symptom severity. The authors interpreted these findings as suggesting a possible common pathway for the development of MCS, despite the fact that the two groups initially experienced exposures that were chemically different. They hypothesized that the relatively greater neurotoxicity or potency of the cholinesterase inhibitors versus mixed low-level solvents might explain the greater symptom severity in the pesticide-exposed group.

Notably, MCS patients frequently report that other individuals simultaneously exposed to pesticides, e.g. family members, friends, or co-workers, did not develop MCS or even experience transient illness. These observations suggest that a subset of the population may be more vulnerable to developing MCS. Some (Black et al., 1990; Simon et al., 1990), but not all (Fiedler et al., 1992) researchers have reported a greater rate of depression and somatization disorder that predated the "initiating" chemical exposure among persons with MCS compared to controls.

A vigorous medical debate has ensued, revolving around whether MCS is: (1) a physiological consequence of chemical exposure in biochemically susceptible individuals; (2) a psychological response to chemical exposure, e.g. a conditioned behavioral manifestation or post-

traumatic stress disorder; or (3) simply the patients' misattribution of some other illness, organically-based or not, to chemical exposures. Conceivably, depression, a prevalent symptom among MCS patients, could be, (1) a physiological or psychological risk factor for the development of MCS; (2) the consequence of patients' having to cope with a perplexing and disabling medical illness; or (3) one of many symptoms of MCS triggered by chemical exposure.

The FSL (Flinders Sensitive Line) rat was developed by selective breeding for increased sensitivity to an OP, so it shares some etiological similarity to patients with MCS who were exposed to pesticides. MCS patients, depressed patients and FSL rats exhibit many of the same intolerances for drugs and many of the same symptoms, as will be described below. The similarities and the extent to which the FSL rat can be considered a model for MCS will be discussed in a final section.

3. An animal model

The model is one with which we have had extensive experience, particularly in research on depressive syndromes (Overstreet and Janowsky, 1991; Overstreet, 1993). Analogies between depressed states and MCS, as well as substance intolerances in FSL rats, first brought our attention to the potential value of this model for experimental studies of MCS.

3.1. Establishment of the model

The model arose from a selective breeding program designed to produce two lines of rats, one with high and one with low sensitivity to the anticholinesterase agent, diisopropylfluorophosphate (DFP) (Russell et al., 1982; Overstreet et al., 1979). The selective breeding program, which was initiated at Flinders University in Adelaide, Australia, utilized decreases in core body temperature, drinking and body weight to measure sensitivity to DFP. A rank-order system was used to give equal weighting to each of the three variables. Rats which had the lowest average ranks were intermated to establish and maintain

the line of more sensitive rats (Flinders Sensitive Line—FSL), while rats which had the highest average ranks were intermated to establish and maintain the line of more resistant rats (Flinders Resistant Line—FRL). Subsequent studies showed that randomly bred Sprague–Dawley rats, from which the lines were originally derived, were not different from the FRL rats. On the other hand, FSL rats were significantly more sensitive to DFP than the other two groups (Russell et al., 1982; Overstreet et al., 1979).

Because early studies ruled out changes in acetylcholinesterase as a mechanism to account for the differential sensitivity of FSL and FRL rats to DFP (Overstreet et al., 1979; Russell and Overstreet, 1987), the effects of other muscarinic agonists on these rats were examined (Overstreet and Russell, 1982; Overstreet, 1986; Overstreet et al., 1986a,b). These studies showed that the FSL rats were more sensitive to pilocarpine, arecoline and oxotremorine than were the FRL rats; this supersensitivity was seen for a variety of responses, including hypothermia, reduced locomotor activity, and suppression of bar-pressing for water reward (Overstreet and Russell, 1982). Biochemical studies indicated that the FSL rats exhibited greater numbers of muscarinic receptor binding sites in the hippocampus and striatum than the FRL rats (Overstreet et al., 1984; Pepe et al., 1988).

As selective breeding progressed, animals were shipped to the University of North Carolina at Chapel Hill (UNC), where colonies were started. Here, young from the FSL and FRL rats were cross-fostered onto pathogen-free mothers in order to establish pathogen-free colonies. Over the last ten generations of FSL and FRL rats bred at the University of North Carolina (S46-S55), there has been no overlap in the two distributions, indicating that the lines are still quite separate for this phenotype.

3.2. Differences in activity after stressors

FSL rats have been reported to have lower locomotor activity than the FRL rats under a number of experimental conditions (Overstreet and Russell, 1982; Overstreet, 1986; Bushnell et al., 1995) but not all (Criswell et al., 1994; Rez-

vani et al., 1994). Those situations which failed to report a difference in activity between the lines involved automated recording systems over long (>1 h) durations in environments to which the animals had become habituated. In contrast, those studies reporting differences in activity employed short exposures (< 5 min) to an open field apparatus. The reduced activity of the FSL rats, at least in males, is commonly seen under baseline conditions in these novel environments (Overstreet and Russell, 1982; Overstreet, 1986), a parallel to euthymic depressed humans (Wolff et al., 1985). In addition, however, locomotor activity is suppressed to an even greater degree when the FSL rats are exposed briefly (2 s) to a mild (1 mA) foot shock (Overstreet, 1986; Overstreet et al., 1989a). In particular, both male and female FSL rats exhibited a similar degree of locomotor suppression after foot shock, even though only the male FSL rats exhibited lower baseline activities (Overstreet, 1986). These data have led to the suggestion that the FSL rat is an animal model of genetic predisposition toward depression, in that dramatically decreased locomotor activity is seen only upon exposure to stressors (Anisman and Zacharko, 1982; Overstreet et al., 1988; Overstreet and Janowsky, 1991).

Results from several other behavioral paradigms are consistent with the view that depressive-like psychomotor retardation symptoms are more apparent in the FSL rats after exposure to stressors. For example, the FSL rats are impaired in active avoidance paradigms compared to the FRL rats (Overstreet and Measday, 1985; Overstreet et al., 1990a, 1992). The reduced ability of the FSL rats to acquire a shock-motivated task is completely consistent with the above results which show a much greater suppression of activity in the FSL rats after exposure to shock. An alternative interpretation, that there are differences in "anxiety" (immobility after shock or in novel environments) between the FSL and FRL rats (Criswell and Breese, 1989) is not supported by experimental results in the elevated plus maze (Schiller et al., 1991).

Another stress-oriented paradigm which has provided important information about behav-

ioral differences between FSL and FRL rats is the forced swim test. Upon initial exposure in a cylinder (18–20 cm diameter) of water (25°C), FSL rats are more immobile than the FRL rats (Overstreet, 1986; Overstreet et al., 1986a; Schiller et al., 1992; Pucilowski and Overstreet, 1993). This exaggerated immobility of the FSL rats is counteracted by chronic but not acute treatment with antidepressants (Schiller et al., 1992; Overstreet, 1993; Pucilowski and Overstreet, 1993). These findings provide further support for the contention that the FSL rat is a useful animal model of depression.

It might be argued that the exaggerated immobility exhibited by the FSL rats in the forced swim test could indicate that they fatigue more easily, or suffer from a loss of energy, as do human depressives. However, psychomotor retardation as such is a more likely explanation for exaggerated immobility because, as indicated above, differences in immobility between the two lines can be seen very quickly in the forced swim test. Similarly, because exposures to the open field test are also brief, fatigue is probably not a factor. Recently, the FSL and FRL rats have been compared in a treadmill task. The FSL rats became exhausted after a shorter period of exercise on the treadmill than the FRL rats (Bailey et al., 1994), a possible indicator of fatigability. Thus, FSL rats appear to fatigue more readily, which may be a parallel to the well known fatigue found in depressed humans.

3.3. Differences in reward-related behaviors

There are also differences in reward-related behaviors between the FSL and FRL rats which are consistent with the proposal that the FSL rats are a model of depression. In an operant bar-pressing task for water reward, the FSL rats learned the task as readily as did the FRL rats, but stabilized at a much lower response rate in the 15-min session even though both groups were deprived of fluid for 23.5 h per day (Overstreet and Russell, 1982). More recently, Bushnell et al. (1995) reported that the FSL rats were much slower to respond in a food-motivated match-to-sample learning task and they became satiated more readily. The FSL rats had to be maintained

at a lower percentage of their free-feeding body weight and have smaller food pellets (37 vs. 45 mg) in order to keep their motivation sufficiently high to complete the session (Bushnell et al., 1995). In some cases, there are not any baseline differences in the FSL and FRL rats in reward-related tasks. For example, the two lines exhibit similar preferences for saccharin solution over water in a two-bottle choice paradigm, but FSL rats exhibit greater decreases in saccharin preference following exposure to stressors (Pucilowski et al., 1993).

3.4. Differences in cognitive behavior

Actively depressed individuals commonly complain about their inability to concentrate and cognitive impairment is frequently associated with the depressed mood. The data on cognitive (learning) impairments in the FSL rats are mixed. Initially, it was found that the FSL rats exhibit much better memories in the passive avoidance paradigm, where a good performance is reflected by the rat remaining stationary (Overstreet, 1986). In contrast, the FSL rat has difficulty acquiring an active avoidance response (Overstreet et al., 1990a, 1992). Since both of these outcomes could be accounted for by the tendency of the FSL rats to inhibit activity after exposure to shock (see above), an appetitive paradigm was used. In this food-motivated matching-to-sample task, the FSL rats exhibited slower responding, but their choice accuracy was not different from that of the FRL rats (Bushnell et al., 1995). Therefore, it must be concluded that the FSL rat does not exhibit the same degree of cognitive impairment as seen in actively depressed humans.

3.5. Differences in sleep

Insomnia, or an inability to sleep, is a symptom commonly reported in depression. There are many forms of insomnia, but the most common complaint of depressives appears to be one of early morning awakening and/or fragmented sleep without being able to return to sleep (Whybrow et al., 1984). It is rather difficult to model these more subjective aspects of sleep. However, there are more complex changes in the sleep-wake cycle of depressed humans which can

be determined by polysomnography studies (Gillin et al., 1979, 1981; Benca et al., 1992). Such studies indicate that sleep in depression can be differentiated from non-depressive sleep (Gillin et al., 1979), and that depression is associated with a reduction in the deeper stages (3 and 4) of sleep, a reduction in the latency to the first episode of rapid eye movement (REM) sleep, and an increase in the amount or density of REM sleep (Kupfer, 1976; Gillin et al., 1979, 1981; Benca et al., 1992). Human depressives are also more sensitive to the effects of cholinergic agonists on REM sleep latency (Janowsky et al., 1994).

Several days of 24 h sleep recordings in FSL and FRL rats have been carried out under baseline undisturbed conditions. These studies indicated that FSL rats exhibited significantly more REM sleep than FRL rats, but there were no differences in the amount of slow wave sleep (Shiromani et al., 1988). In addition, there was a significantly shorter interval between REM episodes in FSL rats. Thus, neither FSL rats nor depressed humans exhibit an overall reduction in sleep time, but both exhibit increases in REM sleep and reductions in REM sleep onset. The selected differences in REM sleep between the FSL and FRL rats have been replicated using a sleep deprivation paradigm (Shiromani et al., 1991) and an automated scoring system (Benca et al., 1993). Interestingly, persistent REM sleep abnormalities have been noted in depressed humans following clinical recovery from depression (Benca et al., 1992), a finding which parallels the "trait" aspects of the FSL rat's "sleep disorder".

3.6. Trait/state considerations

It has been recognized for some time that many biological changes seen in depressed individuals are present only while they are actively depressed (state changes), while others can be observed even after the individual has recovered (trait characteristics; Janowsky et al., 1994). Therefore, if the FSL rat is a true genetic animal model of depression, then it should not be "depressed" all the time. Most individuals suffering from depressive disorders have long periods of essentially normal behavior between episodes. Study of the FSL animals under various manipu-

lations may help to understand how genetic and environmental variables interact to produce depressive-like phenomena. Thus, the REM sleep changes observed in FSL rats may reflect an underlying trait predisposing to depression (Benca et al., 1992; Janowsky et al., 1994). Reduced activity can be seen in FSL rats under baseline conditions, just as has been observed in euthymic depressed patients (Wolff et al., 1985); however, the reduced activity in both FSL rats and depressed humans is much more prominent after exposure to stressors. Finally, studies suggest that superimposing the chronic mild stress paradigm upon this genetic model has resulted in data indicating that FSL rats appear to be more "anhedonic" than FRL rats since the former exhibit a significantly greater decrease in saccharin preference (Pucilowski et al., 1993). An unanswered question is whether the anhedonia lasts for a longer period of time in the FSL rats.

In sum, the FSL rats and depressed humans exhibit a large number of behavioral and physiological similarities (see Overstreet, 1993, for a more detailed description).

3.7. Drug interactions

Clinical observations suggest that MCS may be initiated by acute or chronic exposure to diverse chemical agents. As described earlier, the FSL rats were selectively bred to have increased responses to the anticholinesterase agent, DFP, so it should not be surprising that they also exhibit increased sensitivity to muscarinic agonists (Overstreet and Russell, 1982; Overstreet, 1986; Schiller et al., 1988; Daws et al., 1991; Overstreet et al., 1992a,b). Notably, there have also been several reports of increased sensitivity to anticholinesterases in human depressives (Janowsky and Risch, 1987; Sitaram et al., 1987; Nurnberger et al., 1989; Gann et al., 1992; O'Keane et al., 1992) and MCS patients (Rosenthal and Cameron, 1991; Cone and Sult, 1992; Miller and Mitzel, 1995). Human depressives are also more sensitive to directly acting muscarinic agonists (Gillin et al., 1991; Gann et al., 1992); according to a recent report, so are children of depressives (Schreiber et al., 1992). At present there are no published data for MCS patients

regarding sensitivity to direct cholinergic agonists in particular, but such agents are among those which many MCS patients say they cannot tolerate.

Knowledge about possible serotonergic alterations in the FSL rats has increased along with the availability of more selective agonists. In early studies, only relatively nonselective drugs were available, and the FSL rats were found to exhibit a greater degree of hypothermia after both *m*-chlorophenylpiperazine (mCPP), a 5-HT_{1B/C} agonist, and cyproheptadine, a nonselective 5-HT (5-hydroxytryptamine) antagonist (Wallis et al., 1988). It was argued that these results favored the hypothesis of a 5-HT₁ supersensitivity in the FSL rats because 5-HT₁ agonists were reported to produce hypothermia while 5-HT₂ agonists induced hyperthermia (Gudelsky et al., 1986). Subsequent studies exploring the hypothermic effects of buspirone and 8-OH-DPAT, selective 5-HT_{1A} agonists, confirmed the 5-HT₁ supersensitivity in the FSL rats (Overstreet et al., 1992). However, using an operant responding paradigm, Schiller (1991) found that the FSL rats were more sensitive to the behavioral suppressant effects of both quipazine, a 5-HT_{2/1C} agonist, and mCPP, a 5-HT_{1B/C} agonist. In addition, he reported preliminary evidence for increases in cortical 5-HT receptors of both subtypes in the FSL rats (Schiller, 1991). Thus, there is considerable evidence for serotonergic supersensitivity in the FSL rats, especially of the 5-HT₁ subtype. This outcome is consistent with much of the evidence suggesting supersensitive serotonergic mechanisms in depressives (Arora and Meltzer, 1989; Arango et al., 1990; Mikuni et al., 1991), but is not consistent with neuroendocrine studies reporting blunted responses to serotonergic agonists, which suggests serotonergic hyposensitivity (Meltzer and Lowy, 1987; Lesch et al., 1990). As yet, there are no data on the effects of selective serotonergic agents in MCS patients, so the similarity between the FSL rats and MCS patients for this parameter cannot be evaluated at present.

If the FSL rat model of depression mimicked the changes in noradrenergic function seen to occur in depressive disorders, then FSL rats

should be supersensitive to beta-noradrenergic agonists and subsensitive to alpha-noradrenergic agonists. To date, there has been no support for this hypothesis in the FSL/FRL rats. The reductions in body temperature and in locomotor activity induced by the beta-noradrenergic agonist salbutamol were similar in the FSL and FRL rats (Overstreet et al., 1989a). Similarly, the reductions in body temperature and in operant responding for water reward induced by the alpha-noradrenergic agonist clonidine were also similar in the FSL and FRL rats (Overstreet, 1989). Thus, even though the studies to date are quite limited, there do not appear to be any marked differences in behaviorally represented noradrenergic function between FSL and FRL rats.

Interactions with the dopaminergic system would be expected because of the dopamine deficiency observed in human depressives (Wise, 1979; Willner, 1983). The prediction is that depressed humans and FSL rats should be supersensitive to the effects of dopamine agonists. The

data collected so far on dopaminergic mechanisms is partially consistent with this hypothesis. The FSL rats are supersensitive to the hypothermic (Crocker and Overstreet, 1991) and aggression-promoting (Pucilowski et al., 1991) effects of apomorphine, a mixed D1/D2 agonist, and quinpirole, a selective D2 agonist. On the other hand, the FSL rats are subsensitive to the stereotypy-inducing effects of apomorphine and quinpirole at similar doses where supersensitivity to the hypothermic effects were seen (Crocker and Overstreet, 1991). In addition, no evidence for differences in dopamine D2 receptors between FSL and FRL rats could be detected in a series of studies (Crocker and Overstreet, 1991). Consequently, it was argued that the opposite changes in sensitivity in the various functions could be related to the way the cholinergic and dopaminergic systems interact to modulate those functions. Both cholinergic and dopaminergic stimulation promote hypothermic and aggressive responses (Cox et al., 1980; Pucilowski, 1987; Ray et al., 1989), but cholinergic stimulation has op-

Table 1
Multiple chemical sensitivity in FSL rats

Compound	Mechanism of action	Responses
Conditions under which FSL rats are more sensitive than FRL rats to drugs		
DFP	Anticholinesterase	Temperature/drinking
Physostigmine	Anticholinesterase	Temperature/activity
Oxotremorine	Muscarinic agonist	Temperature/activity
Pilocarpine	Muscarinic agonist	Temperature/activity
Arecoline	Muscarinic agonist	Temperature/activity
Nicotine	Nicotinic agonist	Temperature/activity
Apomorphine	Dopamine D1/2 agonist	Temperature
Quinpirole	Dopamine D2 agonist	Temperature
Raclopride	Dopamine D2 antagonist	Catalepsy
mCPP	5-HT-1B agonist	Temperature/activity
8-OH-DPAT	5-HT-1A agonist	Temperature
Buspirone	5-HT-1A agonist	Temperature
Diazepam	Benzodiazepine agonist	Temperature/activity
Ethanol	Multiple (GABA, 5-HT)	Temperature
Conditions under which FSL rats are less sensitive than FRL rats to drugs		
Scopolamine	Muscarinic antagonist	Activity
Apomorphine	Dopamine D1/2 agonist	Stereotypy
Quinpirole	Dopamine D2 agonist	Stereotypy
MK-801	NMDA antagonist	Temperature

Table 2
Effects of blocking drugs on ethanol-induced hypothermia in Flinders Sensitive and Resistant rats

Compound	Dose	Decrease in temperature (°C, mean \pm S.E.M.)		
		FSL rats	FRL rats	Difference
Experiment 1				
Ethanol (E) only	3 g/kg	2.8 \pm 0.5	1.6 \pm 0.5	1.2
E + scopolamine	1 mg/kg	2.6 \pm 0.5	1.6 \pm 0.4	1.0
E + bicuculline	2 mg/kg	2.7 \pm 0.6	1.6 \pm 0.5	1.1
Experiment 2				
Ethanol only	3 g/kg	2.3 \pm 0.5	1.2 \pm 0.4	1.1
E + verapamil	10 mg/kg	3.0 \pm 0.6*	1.8 \pm 0.5*	1.2
E + nicardipine	10 mg/kg	2.2 \pm 0.5	0.9 \pm 0.6	1.3
E + haloperidol	0.5 mg/kg	2.1 \pm 0.5	1.0 \pm 0.5	1.1
E + naltrexone	2 mg/kg	2.3 \pm 0.7	1.0 \pm 0.5	1.3
E + pindolol	1 mg/kg	1.9 \pm 0.8	1.0 \pm 0.5	0.9
E + mecamlamine	5 mg/kg	5.6 \pm 1.2*	2.8 \pm 0.8*	2.8**
E + hexamethonium	5 mg/kg	3.3 \pm 1.1*	1.9 \pm 0.9*	1.2
Experiment 3				
Ethanol only	3 g/kg	2.9 \pm 0.3	1.7 \pm 0.3	1.2
E + idazoxan	3 mg/kg	5.2 \pm 0.3*	4.0 \pm 0.3*	1.2
E + nicotine	0.4 mg/kg	4.0 \pm 0.4*	2.5 \pm 0.3*	1.5**

*Significantly greater than ethanol only; potentiated hypothermia.

**Greater difference in temperature than with ethanol only.

posite effects to dopaminergic stimulation in the modulation of activity and stereotypy (Fibiger et al., 1970; Klemm, 1989).

In addition to the above drugs which interact selectively with specific neurotransmitter receptors, the FSL and FRL rats are differentially sensitive to the effects of several other pharmacological agents, as summarized in Table 1. However, as with the case of dopamine agonists, the differential effects are observed only for some actions of the drugs, not for all. For example, ethanol induces a greater hypothermia in the FSL rats, but not a greater intoxication (Overstreet et al., 1990b). Similarly, diazepam produces greater behavioral suppressant effects in the FSL rats (Pepe et al., 1988), but the anxiolytic effects of diazepam in the two lines are comparable (Schiller et al., 1991).

We pretreated FSL and FRL rats with antagonists selective for specific neurotransmitters in an attempt to determine if the differences in

ethanol-induced hypothermia in the two lines were related to particular neurotransmitter differences. The results of this study (previously unpublished) are shown in Table 2. Although some compounds altered the hypothermic effects of ethanol, there were parallel changes in the two lines and no agent reduced the 1°C difference between the two lines. Surprisingly, the nicotine antagonist, mecamlamine, dramatically potentiated the hypothermic effects of ethanol more in the FSL than the FRL rats. These findings provide extensive replication of the previously reported differences in ethanol-induced hypothermia between the FSL and FRL rats (Overstreet et al., 1990b), but have failed to elucidate the mechanism underlying this differential effect.

In summary, it is quite clear that the FSL rat is more sensitive to a variety of chemical agents in addition to the anticholinesterase for which they were selectively bred. In this regard, the FSL rat is, in part, analogous to MCS patients who

become more sensitive to a range of agents following exposure to OP anticholinesterases. The extent of the similarity between the FSL rats and MCS patients, on one hand, and human depressives and MCS patients, on the other, will be evaluated in the next section.

4. Mechanisms of action

As Table 3 summarizes, the behavioral features of individuals with MCS and those of depressed patients and FSL rats are strikingly similar in regard to weight, appetite, activity and stressability, hedonia, and sleep. A closer look at Table 3 suggests several studies that might be carried out in MCS patients to test further the extent of the associations among the three groups. For example, polysomnographic recordings of sleep in asymptomatic MCS patients would be particularly informative, especially since there is evidence that the REM sleep changes seen in depressed patients may be a trait marker of this disorder (Benca et al., 1992; Janowsky et al., 1994). So far, information about sleep in MCS patients is of a more subjective nature. However Bell (1995) has recently reported a decrease in slow wave sleep in individuals with odor intolerances, a finding found in human depressives but not FSL rats. Since REM sleep alterations can also be related to altered cholinergic mechanisms in general (Shiromani et al., 1987; Janowsky et al., 1994), a finding of REM sleep changes in

MCS patients would suggest that altered cholinergic mechanisms might underlie abnormal sensitivity to chemicals. Such a finding would also be consistent with a cholinergic hypothesis as one possible explanation for the similarity between the MCS patients and depressives.

Another similarity between MCS and depression is that there are many more females than males expressing the symptoms (Table 3). Twice as many females than males report depressive symptoms, but the incidence of bipolar illness is equal in the genders (Goodwin and Jamison, 1990). The ratio of female to male MCS patients is even higher, reaching 4/1 in some studies (Miller and Mitzel, 1995). The information on cholinergic sensitivity in the female and male FSL rats cannot easily be related to the human data, because sensitivity is a continuous variable and neither the female nor the male populations overlap with their FRL counterparts (Overstreet, 1993). However, adult female FSL rats are more sensitive to cholinergic agonists than their male counterparts (Netherton and Overstreet, 1983). The greater sensitivity of adult females to cholinergic agonists might therefore contribute to the greater incidence of depression (Overstreet et al., 1988) and MCS in this gender.

Given the behavioral similarities between MCS and depressed patients (Table 3), one could predict that depressed patients might be hypersensitive to various drugs. Unfortunately, as described in Table 4, there is not much information

Table 3
Comparison of characteristics and behavioral features of MCS patients, FSL rats and depressed patients

Measure	MCS patients	FSL rats	Depressed patients
Weight	Up or down	Down	Up or down
Appetite	Up or down	Down	Up or down
Blood pressure	Up or down	ND	Up or down
Food craving	++	+	+
Sleep disturbances	+++	++	+++
Loss of drive	+++	+++	+++
Reduced activity	+++	+++	+++
Cognitive disturbance	+++	+/-	+++
Gender ratios (F/M)	4/1	F > M	2/1

ND, not determined

Table 4
Comparison of drug sensitivity in MCS patients, FSL rats and depressed patients

Compound	MCS patients	FSL rats	Depressed patients
Anticholinesterases	+++	+++	+++
Solvents, etc.	+++	ND	ND
Ethanol	+++	++	+
Nicotine	+++	++	+
Xanthines	+++	ND	ND
Foods	+++	ND	ND

ND, not determined.

concerning the sensitivity of depressed individuals to the range of drugs reported to cause problems in MCS patients, other than their supersensitivity to anticholinesterases and cholinergic agonists (Janowsky et al., 1994). There is somewhat more evidence for a general increase in sensitivity to drugs in the FSL rats (Tables 1 and 4). It is particularly noteworthy that the FSL rats are more sensitive to both alcohol (Overstreet et al., 1990b) and nicotine (Schiller and Overstreet, 1993). The information on the effects of alcohol and nicotine in depressed patients is more complex, as implied by the question mark in Table 4. While we are not aware of any studies specifically stating that depressed patients report intolerances for alcohol and/or nicotine, there are much data related to the interaction of depression with primary alcoholism on one hand (e.g. Schuckit, 1986; Kendler et al., 1993; Maier et al., 1994) and to the interaction of smoking with depression on the other (Breslau et al., 1991; Glassman, 1993).

It should be stressed that FSL rats are also less sensitive to certain drugs (Table 1) and that depressed patients exhibit blunted hormonal responses to a number of drugs affecting serotonergic and noradrenergic mechanisms (Meltzer and Lowy, 1987). Therefore, there is a need to collect more data from depressed individuals and FSL rats on their sensitivities to a broader range of chemicals. If the cholinergic system is a link between the three conditions, then it would be predicted that both FSL rats and depressed individuals would be more sensitive to such drugs. Also needed is more data on depressed individuals and FSL rats with respect to the triggering

of symptoms by chemical or food exposures (Table 4).

Although we have emphasized the possibility of a cholinergic link between MCS patients, depressed patients, and FSL rats, other neurotransmitter systems may be involved. Serotonin (5-hydroxytryptamine; 5-HT) has been implicated in depression (Meltzer and Lowy, 1987) and recent experiments on the Flinders rats suggest that serotonergic mechanisms may play an important role in some of their altered behaviors (Overstreet et al., 1994). We can only speculate about the role of serotonergic mechanisms in MCS patients, as there are no data. However, given the wealth of information on serotonergic mechanisms in depressed patients, it should be possible to design and conduct appropriate experiments to address this possibility in MCS patients.

A somewhat more complex neurotransmitter model proposes that the various neurochemical systems interact with one another and that abnormal behavioral states may arise from an alteration in one system which creates an imbalance in interactions with others. The original concept proposed by Janowsky et al. (1972) suggested that depression and mania were the consequence of imbalances between the noradrenergic and cholinergic systems, with depression being associated with relative cholinergic overactivity and mania being associated with relative noradrenergic overactivity (see also Fibiger et al., 1970). This model could account for some of the effects observed in the FSL rats following administration of noncholinergic drugs. For example, FSL

rats are more sensitive to the hypothermic effects of dopamine agonists, but less sensitive to their stereotypy-inducing effects (Table 1). Since dopaminergic and cholinergic systems work in parallel to regulate temperature but in opposition to regulate activity and stereotypy, an overactive cholinergic system could account for the findings with the dopamine agonists (Overstreet, 1993).

Another type of mechanism which could underlie all three conditions is a change in second messenger rather than neurotransmitter functions. Several investigators have proposed that changes in G proteins, cyclic AMP or other second messenger systems may be involved in depression (Wachtel, 1989; Lesch and Manji, 1992; Avissar and Schreiber, 1993). It has been suggested that the functional muscarinic responses in the FSL and FRL rats are too divergent to be explained by the relatively small differences noted in muscarinic receptors (Overstreet, 1993). If correct, such a hypothesis may more easily account for the pervasiveness of the chemical sensitivity described in MCS patients, which involves many classes of chemical compounds besides those having direct effects on neurotransmitter systems. Differences in second messengers could be hereditary or induced by exposure to chemical agents. Further study of FSL rats, MCS patients, and depressed patients using diverse approaches is needed to obtain a clear picture of the mechanisms that may underlie MCS.

5. A proposal for future studies

In conclusion, we propose that the characteristics of the animal model we have described are sufficiently analogous to MCS to warrant its use in testing hypotheses about the etiology and mechanisms of action involved in the syndrome. An example of the type of experimental protocols suggested by this review is the study of FSL and FRL rats after exposure to volatile solvents and other chemicals to which MCS patients report intolerance. If FSL rats do exhibit increased sensitivity to a wide variety of chemical agents, then treatment approaches could be attempted

using antidepressant drugs, for example. It should be emphasized that proposing antidepressant treatment does not presume that depression is the cause of MCS; quite the reverse might be true. For example, exposure to OPs might augment cholinergic sensitivity, leading to both MCS and depression. The possibility that increased cholinergic sensitivity might underlie both MCS and depression suggests further experiments in these patient groups. Is there a subset of depressed patients who report intolerance to varied substances? Do these same patients exhibit a greater sensitivity to cholinergic agents? Would this subset of depressed patients benefit from avoidance of certain drugs and environmental exposures? Do MCS patients have altered cholinergic responsivity?

These experimental approaches can provide information which is not obtainable by other methods and which, therefore, could add significantly to our knowledge of a disabling syndrome, one which is strikingly similar to the illness reported among Gulf War veterans (Miller, 1994b; Miller and Mitzel, 1995). As Dr. Louis W. Sullivan, then U.S. Health and Human Services Director, stated at an Experimental Biology meeting in 1990:

humanely conducted animal research is critically important in our search for cause and treatments for AIDS, cancer, Alzheimer's disease, schizophrenia and other diseases, just as it was critically important for virtually every major biomedical discovery in the past.

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Toxicology 111 (1996) 135–145

TOXICOLOGY

Proposed animal neurosensitization model for multiple chemical sensitivity in studies with formalin

Barbara A. Sorg^{*a}, James R. Willis^b, Thomas C. Nowatka^a, Catherine Ulibarri^a,
Ronald E. See^b, Hal H. Westberg^c

^a*Program in Neuroscience, Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, Washington 99164-6520, USA*

^b*Department of Psychology, Washington State University, Pullman, Washington 99164-6520, USA*

^c*Department of Civil and Environmental Engineering, Washington State University, Pullman, Washington 99164-6520, USA*

Abstract

A potentially promising line of animal research relevant to multiple chemical sensitivity (MCS) is that of sensitization in the central nervous system (CNS), particularly limbic pathways in the brain. Sensitization is the progressive and enduring enhancement in behavioral and neurochemical responses that occurs after repeated exposure to psychostimulants or environmental stressors. Since the onset and progression of sensitization has many parallels with that of MCS, it has been proposed that MCS may be initiated through a mechanism similar to the sensitization of CNS components occurring in the rodent. To test this hypothesis, female Sprague–Dawley rats were exposed to formalin vapors (FORM, 11 ppm) or water vapor (control) 1 h/day for 7 days. The next day, a saline injection was given followed by a cocaine injection (15 mg/kg, i.p.) 24 h later, and locomotor activity was monitored. Animals pretreated with repeated FORM inhalation demonstrated a significantly enhanced locomotor response to cocaine compared to controls, an indicator that specific limbic pathways may have been sensitized. At 4 weeks of withdrawal from FORM exposure, a subset of animals remained sensitized to a cocaine challenge. No differences were found between groups after a saline injection. In a second experiment, animals were screened prior to FORM or water exposure for their response to a novel situation, a measure believed to reflect an animal's general responsiveness to stimuli. Rats were divided into high responders (HR) or low responders (LR), based on their locomotion in a novel cage. Results from three behavioral tests demonstrated that HR and LR were differentially affected by exposure to FORM. In a passive avoidance test, HR and LR appeared to be different in their distribution of responses, while HR and LR responses in the FORM group were nearly identical. On the elevated plus maze test of anxiety, HR spent more time on the open arms than LR in both treatment groups, with significant differences between HR and LR in the FORM, but not water, treated group. On a hot plate test to measure nociceptive levels, no differences occurred between HR and LR in the control group, whereas nociception of LR tended toward an increase compared to HR in the FORM-exposed group. Results from the second experiment suggest that the effects of FORM exposure may be obscured by examining behavior in a heterogeneous population (HR and LR). This approach using animal models may help define neural substrates that mediate the amplification of responses of a subpopulation of individuals to chemicals in the environment.

Keywords: Chemical sensitivity; Formaldehyde; Formalin; Mesolimbic system; Sensitization

*Corresponding author, Tel.: 509-335-4709; Fax: 509-335-4650.

1. Introduction

Multiple chemical sensitivity (MCS) is an ill-defined and controversial disorder in humans attributed to exposure to a wide range of volatile organic compounds. Individuals who develop sensitivity to chemicals report an array of symptoms, including depression, fatigue, headaches, gastrointestinal problems, irritability, muscle aches, and memory and concentration difficulties, among many others (Ashford and Miller, 1991). The controversy surrounding MCS partly stems from the question of how amplification of sensitivity to chemicals can occur, since symptoms have been reported after exposure to extremely low levels of chemicals that do not affect the general population. Bell et al. (1992) have noted that MCS strongly resembles the phenomenon of sensitization in the central nervous system (CNS), and have proposed that amplification of responses in MCS individuals develops via CNS sensitization. Sensitization is defined as the progressive and enduring enhancement in behavioral and neurochemical measures after repeated, intermittent exposure to a variety of stimuli, most commonly; psychostimulant drugs and environmental stressors are used (Antelman, 1988). Several features of sensitization appear parallel to those of MCS, including the progressive increase in sensitivity to drugs/chemicals (Post and Weiss, 1988; Bell, 1994); the apparent permanence of sensitivity (Robinson and Becker, 1986; Ashford and Miller, 1991); the greater sensitivity of females vs. males (Camp and Robinson, 1988; Miller, 1994); and the spreading of sensitization in response to stimuli other than the initial stimulus used to induce sensitization (as with cross-sensitization between psychostimulants and stress) (Antelman et al., 1980; Miller, 1994).

Sensitization is typically initiated by repeated administration of psychostimulants such as cocaine and amphetamine. Rodents sensitized to these drugs or to environmental stressors demonstrate an augmentation in locomotor activity in response to a subsequent challenge of psychostimulants (see Kalivas and Stewart, 1991 for review). One major mechanism contributing to the manifestation of locomotor sensitization in-

volves the mesolimbic dopamine system, including dopamine perikarya in the ventral tegmental area and their projections to the nucleus accumbens. Extracellular dopamine levels are enhanced in the nucleus accumbens in animals sensitized to psychostimulants, and this enhancement is believed to partially contribute to the sensitized behavioral response (Robinson et al., 1988; Kalivas and Duffy, 1990; Parsons and Justice, 1993).

Due to the vast literature implicating the mesolimbic dopamine system in the phenomenon of behavioral sensitization and the strong parallels between sensitization and MCS, the present study focused on developing an animal model of MCS by examining behavioral sensitization in animals repeatedly exposed to formalin (FORM) vapors. Formaldehyde and FORM are among the most ubiquitous volatile organic compounds found in indoor air, present in hundreds of common products such as paper, insulation and wood products. Formaldehyde is among the more problematic chemicals for individuals with MCS (Ashford and Miller, 1991). To test for behavioral sensitization, a subsequent cocaine challenge was given and the locomotor response was measured, referred to as "cross-sensitization" because two different stimuli are involved. In addition, since no animal model for MCS has been established, further behavioral testing was performed to obtain a profile of the changes that accompany repeated exposure to FORM vapors. Three tests were chosen based on observations of decreased memory and concentration, increased anxiety, and increased somatization in individuals with MCS (Ashford and Miller, 1991). A memory task using the passive avoidance test, an anxiety test using the elevated plus maze, and a hot plate test for nociception were used to examine animals repeatedly exposed to FORM.

2. Methods

2.1. Drugs and reagents

Cocaine hydrochloride (15 mg/ml) was dissolved in 0.9% saline. Corticosterone (CORT) levels were determined with a radioimmunoassay kit

(Diagnostic Products Corporation, Inc., Los Angeles, CA).

2.2. Animals

Female Sprague–Dawley rats were group-housed three or four per cage in a temperature- and humidity-controlled room with food and water available ad libitum except for the time during which they were placed into the exposure chambers. The room was maintained on a 12-h light/dark cycle, with lights on at 0700 h.

2.3. Experimental apparatus

Rats were placed into a chamber as shown in Fig. 1. Air was pumped into a 0.5% FORM solution or distilled water (control condition), and the vapor above the solution was passed through the chamber. Formaldehyde levels tested during the 1-h exposure were 11.0 ± 1.3 ppm (mean \pm S.D.) between 10–20 min and 11.3 ± 0.3 ppm in the last 10 min of the 1-h period of exposure.

Locomotor activity was monitored in square Plexiglas cages measuring 40 cm² containing eight photocells in each direction 2 cm from the floor. The photocell cages were located in individual wooden boxes with separate lighting (10 W) and a fan.

The elevated plus maze consisted of a “plus”-shaped platform made of black opaque Plexiglas which was 10 cm in width and 50 cm in length, creating a 10 × 10 cm “neutral zone” (see below). The plus maze was elevated 50 cm from the floor. Two of the arms were enclosed with black Plexiglas walls 40 cm high, with no ceiling.

The passive avoidance apparatus (Stadler Grason, W. Concord, MA) consisted of two chambers (23 × 20 cm) connected by a removable black opaque sliding door between the chambers. One chamber was clear Plexiglas with additional lighting, the other side consisted of black opaque Plexiglas. The shock delivered was 0.4 mA for 3 s.

Nociception was measured using a hot plate (Columbus Instruments, Columbus, OH). The temperature was set at a constant 52°C for testing.

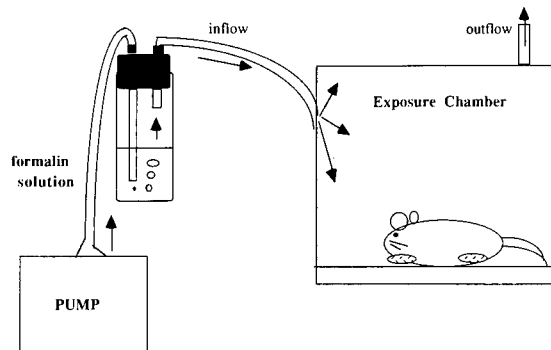


Fig. 1. Schematic of chemical exposure chamber. Rats were placed three per cage into the exposure chamber. Air was pumped into a 0.5% formalin solution and vapor above was passed through a liquid trap before entering the chamber. Outflow was vented to a fume hood.

For measuring CORT levels after a stressful stimulus, animals were administered restraint stress. Restraint consisted of a Plexiglas bottom piece with two padded wire mesh screens that wrapped around the animal and secured with Velcro fasteners.

2.4. Treatment protocol

Two experiments were performed (refer to Table 1 for time-table of experiments). The first experiment was done to test whether animals that were repeatedly exposed to FORM would demonstrate cross-sensitization to cocaine-induced locomotion. For the first experiment, rats were placed in groups of three into the exposure chambers for 1 h/day for 7 days. The next day, rats were allowed to habituate for 1 h in the photocell apparatus, a saline injection was given (1 ml/kg, i.p.), and locomotor activity was monitored for an additional 1 h. The following day, the animals' response to cocaine hydrochloride (15 mg/kg, i.p.) was monitored in the same manner except that locomotion was measured for 2 h. One month later, the cocaine-induced motor-stimulant response was again measured as described to determine if the effects of FORM exposure produced a long lasting cross-sensitization to cocaine.

The second experiment was performed to obtain a broader behavioral profile of animals ex-

Table 1
Time-table for experimental procedures

Experiment 1												
Day	1	2-8	9	10	11-36	37	38					
	Novel Screen	FORM exposure	Saline	Cocaine	Home cage (no treatment)	Saline	Cocaine					
Experiment 2												
Day	1	2-8	9-11	12	13	14-19	20	21	22-29	30	31-35	36
	Novel screen	FORM exposure	Home cage	Plus-maze	PA train ^a	Home cage	PA test ^a	Noci-ception	Home cage	Cocaine	Home cage	CORT

^aPassive avoidance training and testing (see text for details).

posed to repeated FORM vapors. For this experiment, animals' locomotor response to a novel situation (photocell apparatus) were measured. Rats were randomly assigned to either the FORM or water-control group. Animals within each group demonstrating a locomotor response to novelty that was higher than the median response were referred to as high-responders (HR), and those responding lower than the median were considered low-responders (LR). This division of HR and LR is believed to be a measure of rats' general responsiveness to the environment (Piazza et al., 1990). One day after this screening procedure, rats were administered repeated FORM exposure as described above. Four days after their last exposure, rats were tested for a variety of behaviors over the next 3 weeks as described below.

For the elevated plus maze test, rats were removed one at a time from their home cage to the testing area. Rats were placed into the center of the plus maze (neutral zone), and the amount of time spent in the open and closed arms was recorded over a 5-min period in the presence of an experimenter who was unaware of the treatment groups. Animals were considered to be in open or closed arms only when all four limbs crossed out of the neutral zone. The elevated plus maze relies on the animal's natural fear of open spaces, and the time spent on the open arms is a measure of general anxiety level (File, 1993). In the present manuscript, open time ratio is re-

ported, which is the time spent on open arms/total time spent in open + closed arms.

To test whether FORM exposure altered memory performance on a passive avoidance task, rats were given a training session and a test session. The training session consisted of the following: the door separating the two chambers was open. Rats were allowed to enter the preferred side (dark side) and to remain there for 5 min. After this habituation period in the dark, rats were placed into the home cage for 5 min. They were then placed into the light side of the chamber, and, upon entering the dark chamber, the sliding door was closed followed by delivery of a 0.4 mA, 3 s shock. The animal was placed back into the home cage for 6 days. On the seventh day, the test session consisted of placing the rat in the light side of the chamber and measuring the latency to enter the dark during a 5-min period.

Assessment of changes in nociception between treatment groups was done using the hot plate test. Animals were placed onto the hot plate apparatus (52°C), and the latency to lick the hind paw was measured. Once this occurred, the rat was immediately placed back into the home cage.

Three weeks after the last daily FORM exposure, rats were placed into the photocell cages to measure their locomotor response to cocaine as described for experiment 1. Rats were allowed to habituate for 1 h prior to cocaine injection. Cocaine hydrochloride (15 mg/kg, i.p.) was ad-

ministered and locomotor activity was monitored for 2 h. Rats were placed in their home cage for 5 days before blood collection.

Serum CORT levels were measured under basal and stressful conditions. Basal levels were measured by removing each animal from its home cage, and within 1 min of removal, rats were decapitated and trunk blood was collected. Animals were retrieved from their home cage by an additional person not involved with the decapitation process. Stress-induced CORT levels were obtained from rats after a 20-min restraint stress. Blood was allowed to coagulate on ice for 30 min and the serum was collected after centrifugation. Serum CORT was determined by radioimmunoassay.

2.5. Formaldehyde level determination and statistical analyses

Formaldehyde levels present in the vapor above the formalin solution were determined by passing the sample air over a cartridge containing dinitrophenylhydrazine (DNPH). The sample flow was generated with a sample pump and monitored with a Matheson flowmeter. After 10-min collections, the cartridges were stored at 5°C until analysis. The sample and blank cartridges were eluted with acetonitrile. Hydrazone concentrations in the eluent were determined by reverse-phase high performance liquid chromatography (HPLC) as previously described (Sirju and Shepson, 1995).

All data were analyzed with a one- or two-way analysis of variance (ANOVA). A Fisher's PLSD test was used for post hoc comparisons following the one-way ANOVA, and a Student's *t*-test was used in the case of significant differences ($P < 0.05$) from the two-way ANOVA.

3. Results

Fig. 2 shows the results of repeated FORM exposure on saline- and cocaine-induced locomotion 1 and 2 days after discontinuing exposures, respectively. While no differences were present between water control and FORM-exposed animals after a saline injection, a significant aug-

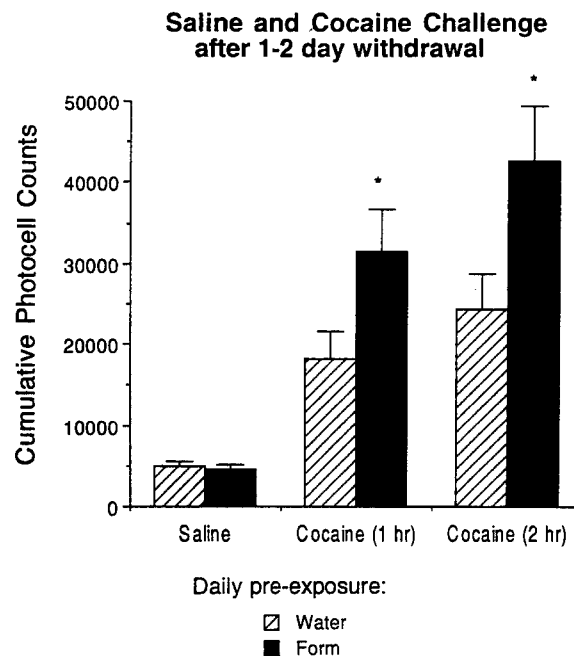


Fig. 2. Cross-sensitization between repeated formalin vapor exposure and cocaine-induced increases in locomotor activity. Values are mean \pm S.E.M. of photocell counts obtained over a 1-h period for the saline day or for both the first hour and second hour after cocaine injection. $N = 9$ /group. For cocaine challenge, 1 h treatment: $F_{1,16} = 4.471$, $P = 0.0505$; for 2 h treatment: $F_{1,16} = 5.268$, $P = 0.0356$.

mentation of cocaine-induced locomotion was found in FORM treated rats compared to controls. When the same animals were allowed to withdraw from FORM exposure for 1 month, no significant enhancement in cocaine-induced locomotor activity occurred. However, since a primary interest in designing an animal model for MCS is an examination of interindividual variability in the expression of sensitization, each animal was plotted separately in Fig. 3. There was a slightly separated subset of the population (4 animals in each group) that demonstrated increased locomotion, with the subset of FORM-exposed rats significantly enhanced above the water-exposed subset. (Mean \pm S.E.M. for water and FORM, respectively, were $59\,509 \pm 3753$ and $74\,156 \pm 2781$; $F_{1,7} = 9.828$, $P = 0.02$). Similar results were obtained in the second experiment (see below and Fig. 4).

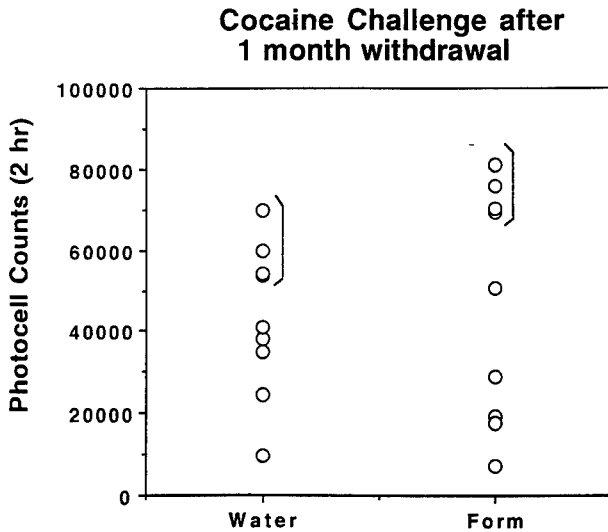


Fig. 3. Individual responses to cocaine challenge after a 1-month withdrawal period. Data are shown as cumulative photocell counts for each rat over a 2-h period from water control- and formalin (FORM)-exposed rats. $N = 9/\text{group}$. The brackets highlight the highest group of responders in each group which are somewhat separated from the remaining animals' responses.

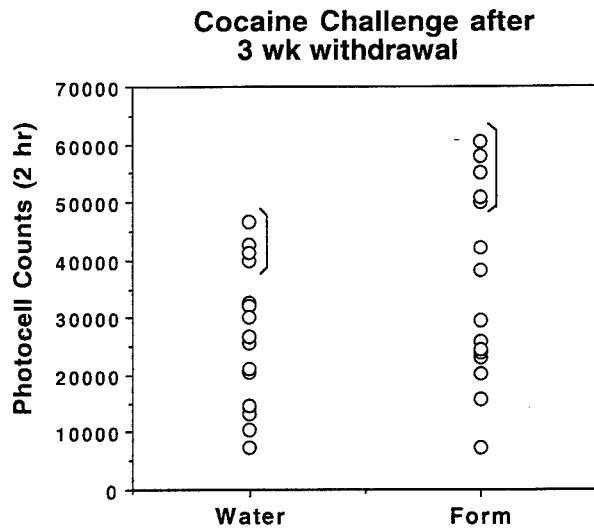


Fig. 4. Individual responses to cocaine challenge after a 3-week withdrawal period from experiment 2 animals. Data are shown as mean \pm S.E.M. of cumulative photocell counts over a 2-h period from water control- and formalin (FORM)-exposed rats. $N = 16/\text{group}$. As in Fig. 3, the brackets highlight the highest group of responders in each group which are somewhat separated from the remaining animals' responses.

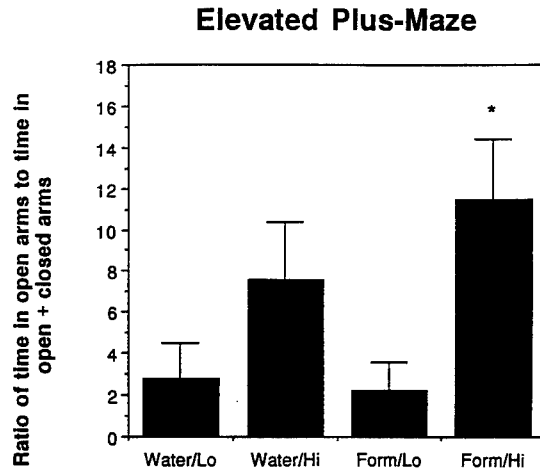


Fig. 5. Ratio of time spent on the open arms to total time spent on open + closed arms of the elevated plus maze. Data are shown as mean \pm S.E.M. of time ratio on open arms of the plus-maze. Water/Lo and Water/Hi are water control LR and HR, respectively, and FORM/Lo and FORM/Hi are formalin-exposed LR and HR, respectively. $N = 8/\text{group}$. A two-way ANOVA revealed that for HR/LR, interaction $F_{1,28} = 9.344$, $P = 0.0049$. * $P < 0.05$, as determined by a Student's t -test between formalin-exposed LR and HR.

In experiment 2, rats were first divided into HR and LR based on their locomotor response to novelty. Mean and standard error for the cumulative photocell counts over a 1-h period in response to novelty in the water group was: HR = 13777 ± 674 , LR = 9104 ± 609 ($P < 0.0001$); and in the FORM group: HR = 13414 ± 638 , LR = 9584 ± 573 ($P < 0.0005$). Fig. 4 shows the motor-stimulant response to a cocaine challenge given 3 weeks after discontinuing repeated daily FORM exposures. Similar to experiment 1, the late withdrawal period produced a subset of animals showing a higher response to cocaine in both groups that were somewhat separated from the other animals. Once again, cocaine-induced locomotion in the FORM pretreated subset was significantly enhanced compared to the subset of control animals. (Mean \pm S.E.M. for water and FORM, respectively, were 42663 ± 1448 and 54912 ± 1977 ; $F_{1,8} = 22.6$, $P = 0.002$). The results suggest that behavioral sensitization may endure in a subpopulation of FORM-exposed rats.

Passive Avoidance

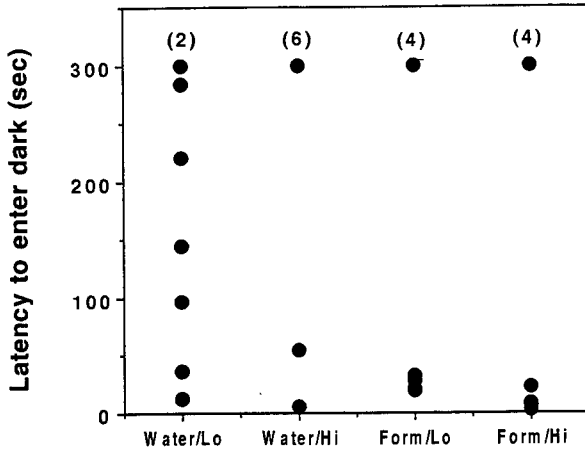


Fig. 6. Individual response on the passive avoidance task. Each circle represents individual animal's response for all latencies < 300 s. The number of animals demonstrating latencies > 300 s is shown in parentheses above a single circle located at the 300-s level.

During the 3-week withdrawal period, three behavioral tests were carried out. Fig. 5 shows the response of animals in the elevated plus maze when tested 4 days after discontinuing exposure. The open time ratio, which is the time spent in

Nociception

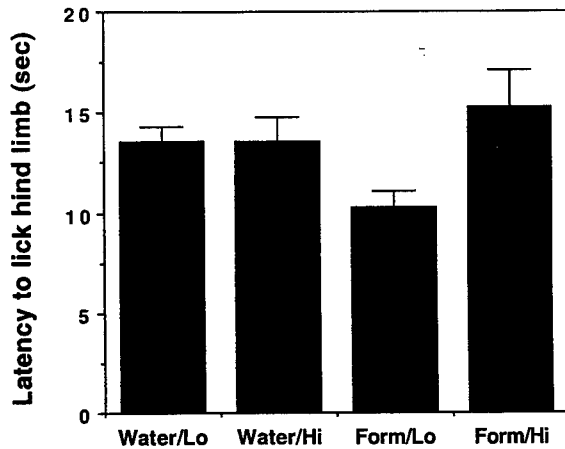


Fig. 7. Hot plate test for nociception. Data are mean \pm S.E.M. of latency to lick the hind limb (in s). See Fig. 5 for description of treatment groups.

Serum Corticosterone

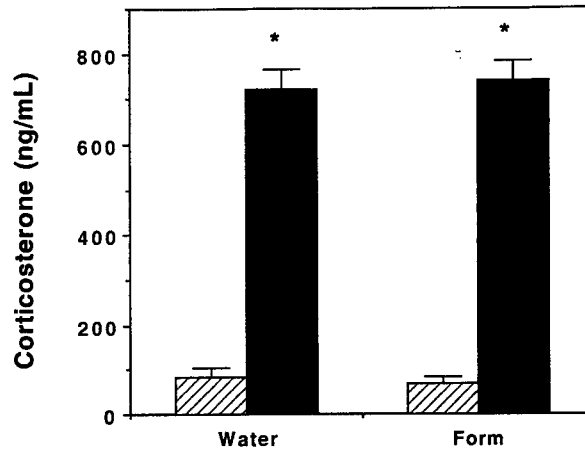


Fig. 8. Basal \square and stress-induced \blacksquare CORT levels. Data are mean \pm S.E.M. of serum CORT levels present basally or after a 20-min restraint stress. For basal values: water, $N = 6$; FORM, $N = 4$. For stress-induced values: $N = 10$ /group. There was a significant effect of stress on CORT levels ($P < 0.0001$).

open/time spent in open + closed arms, reflects general anxiety levels (File, 1993). The open ratio time was greater in HR than in LR, with no main affect of water or FORM treatment. Subsequent analysis between HR and LR within each group revealed that no significant differences were present between HR and LR in the water control group, but that, within the FORM pretreated group, HR were significantly elevated above LR in the time spent on open arms.

Shown in Fig. 6 are the results from the passive avoidance task. Animals did not demonstrate differences in mean latencies to enter the dark among the four groups (data not shown). However, many animals demonstrated latencies of > 300 s; that is, they did not enter the dark in the allotted 300 s time period. Initial chi-square analysis of the proportion of animals that displayed a latency of > 300 s showed no significant differences among groups. However, it is apparent from the manner in which Fig. 6 is presented that the distribution of latencies in HR and LR were different from each other in the water control group, whereas the distribution of latencies between HR and LR were nearly identical in the

FORM pretreated group. It is possible that with additional animals, a significant difference would have been revealed using the chi-square analysis.

Results from the hot plate test for nociception are shown in Fig. 7. No significant differences among groups were present ($P = 0.083$), although there was a trend for FORM-pretreated LR to demonstrate decreased latency to hindlimb licking compared to HR in the same group ($P = 0.045$).

Serum CORT levels were determined both basally and after a 20-min restraint stress (Fig. 8). While a robust increase in CORT levels occurred after the stressor in both groups, no significant differences between FORM and control groups were present for either basal or stress-induced levels.

4. Discussion

4.1. Cross-sensitization between formalin and cocaine

The major finding of this study demonstrates that repeated daily FORM exposure (1 h/day for 7 days) produced a cross-sensitization with cocaine-induced locomotor activity early after withdrawal (2 days), while only a subset of the FORM-exposed animals appeared to remain sensitized 3–4 weeks later. This finding suggests that, initially after repeated exposure to FORM, the entire population was affected with regard to sensitization of pathways involved in locomotor output, but these changes endured only in a subset of animals, while the majority of rats returned to non-sensitized levels.

These findings are in agreement with previous reports of enhanced locomotor activity in response to another dopamine agonist, apomorphine, after repeated low level toluene exposure (von Euler et al., 1991, 1993). The present findings imply that mesolimbic circuitry responsible for maintaining sensitized locomotor responding to a cocaine challenge is involved in FORM-induced sensitization. It can be speculated that those animals which appear to remain sensitized after a 3–4 week withdrawal period exhibit differences in their mesolimbic dopamine function-

ing compared to those animals demonstrating a much lower response. More recent data examining cocaine-induced sensitization may provide an additional explanation to account for the differences between repeated daily FORM-treated animals that demonstrate behavioral sensitization and those that do not at the later withdrawal periods. It is often found that a portion of rats treated with daily cocaine (10–30%, unpublished observations) will not demonstrate behavioral sensitization. Bell (1994) has shown that sensitivity to excitatory amino acids in the nucleus accumbens may be a critical factor in the manifestation of behavioral sensitization. Direct microinjection of the excitatory amino acid agonist, AMPA, into the nucleus accumbens results in enhanced locomotor activity in rats demonstrating behavioral sensitization compared to those rats given repeated daily cocaine but not displaying behavioral sensitization. Thus, in FORM-exposed animals, a further neurochemical distinction between rats exhibiting sensitized and non-sensitized behavior may be possible.

One possibility for the finding that FORM exposure cross-sensitizes to cocaine-induced locomotion is that inescapable exposure to FORM is serving as a stressful stimulus. It is a well-documented phenomenon that stress and psychostimulants cross-sensitize to produce behavioral sensitization (Antelman et al., 1980; Herman et al., 1984; Robinson et al., 1985; Sorg and Kalivas, 1991). It remains to be tested whether FORM-induced effects are acting via those pathways utilized by stress-induced sensitization, or are producing effects either independently of or in concert with stress. Whether the effects of FORM exposure occur through stress-induced vs. chemical-induced pathways may have implications for prevention and/or treatment strategies in chemically-exposed individuals.

4.2. Individual differences in behavioral responses

The subset of animals from experiment 2 that displayed the highest motor-stimulant response to cocaine were comprised mainly of the HR group (4/4 for control; 4/5 for FORM-treated group). These results are in agreement with previous studies demonstrating a positive correla-

tion between locomotor response to novelty and locomotor response to an acute cocaine injection (Hooks et al., 1991). Neurochemical differences between HR and LR have been reported within mesolimbic circuitry (Hooks and Kalivas, 1994). In naive rats, HR demonstrate a higher locomotor response to both dopamine and AMPA, but not to a μ -opioid agonist microinjection into the nucleus accumbens. Whether HRs or a subset of HRs are indeed those animals which become more aversive to FORM is unknown, but is currently being tested in additional studies in this laboratory.

Results from the behavioral studies testing for memory, anxiety and nociceptive levels indicate that HR and LR are differentially affected by FORM exposure. Although a trend for HR to spend more time on the open arms of the plus maze than LR was observed in the control group, these same differences were more pronounced in the FORM-pretreated animals. The results suggest a greater separation of responses among FORM-exposed rats. The increased amount of time spent on the open arms of the plus-maze may be due to increased activity levels. A comparison of the locomotor response to novelty with the ratio of time on open arms revealed a positive correlation between the two measures ($N = 32$; $R = 0.539$; $p < 0.002$). However, this difference in spontaneous locomotion does not fully explain the differences between treatment groups, since analysis of [open time ratio/locomotor activity in response to novelty] still yields significant differences between LR and HR in the FORM-pretreated group ($P = 0.0266$), but not in the water control group ($P = 0.3691$).

In the passive avoidance task, it is interesting to note that in the control group, LR exhibited a greater distribution of latencies compared to HR in this group. The FORM-exposed animals displayed a more dichotomous distribution, with nearly identical responses between HR and LR. In the FORM-pretreated group, for both HR and LR, one-half the rats entered the dark chamber rapidly (within 33 s) and the other half did not enter at all (> 300 s). Therefore, a separation of responses occurred in both HR and LR, and thus the major differences in this task were noted

between LR groups of control and FORM-exposed rats.

The hot plate test for nociception was somewhat similar to the plus maze results, in that there was a strong tendency for FORM-exposed HR and LR to demonstrate differences while no such distinction was noted between HR and LR in the control group.

These latter results, taken at face value, do not appear to be in general agreement with reports from MCS individuals. Decreases in memory and concentration, increases in anxiety, and possible increases in nociception (increased somatization) are common symptoms of MCS (Miller, 1994). In the present study, no clear-cut distinctions between FORM- and water-pretreated groups were demonstrated that are parallel to reports from MCS patients. One possible explanation is that all behavioral testing in the present studies was conducted under basal conditions. Since many MCS patients report symptoms only after re-exposure to chemicals (Ashford and Miller, 1991), it may not be possible to distinguish changes in the absence of a FORM challenge. A second explanation for some of the results is that the passive avoidance procedure is a test of memory that may include a component of learned anxiety. Since increased somatization has been reported in MCS individuals, the possibility remains that some FORM-exposed animals may demonstrate increased memory to avoid the chamber associated with shock due to increased somatization.

Of note is the finding that only subpopulations of FORM-exposed animals were behaviorally altered. This situation is likely to more closely reflect the heterogeneity found in the human population, in which induction of chemical sensitivity is believed to be dependent on a combination of genetic factors and environmental stimuli. Considering the differences between the human and the rat, it may be unrealistic to expect high face validity for such a model. However, the predictive validity may prove to be of far greater importance with regard to determining underlying neurochemical changes, such as those associated with sensitization of limbic structures influencing complex behaviors like anxiety and

memory. Structures that have been strongly implicated in these behaviors are the amygdala and hippocampus. Amplification of symptoms in MCS may originate via sensitization of multiple limbic pathways, including the amygdala and hippocampus, which send excitatory amino acid projections to the nucleus accumbens (DeFrance et al., 1980; Christie et al., 1987; Mogenson et al., 1993). Indeed, the amygdala and hippocampus are important for inducing behavioral sensitization to psychostimulants (Yoshikawa et al., 1993; Kalivas and Alesdatter, 1993), and repeated pesticide exposure induces kindling, a form of sensitization, in the amygdala (Gilbert, 1995). Thus, the amplification process may involve multiple components impacting many behaviors that are altered after chemical exposures.

4.3. Basal and stress-induced CORT levels

No differences were present in either basal CORT or in restraint-stress-stimulated increases in CORT levels. However, the approximate 10-fold increase in CORT levels after restraint suggests that this may have been perceived as an intense stressor. These results point to the need for examining a milder stress, such as novelty-induced CORT, in which levels increase approximately three times (Piazza et al., 1991). In addition, it is possible that differences exist between HR and LR; however, the division of animals into basal and stress groups precluded us from examining the response of HR vs. LR.

5. Summary

Repeated daily FORM exposure produced a behavioral cross-sensitization with cocaine-induced locomotor activity within 2 days after discontinuing daily exposures. After a 3–4 week withdrawal period, cocaine-induced locomotion was enhanced in only a subgroup of FORM-treated animals. These data suggest that FORM exposure may initially produce a sensitization that is only transient in most animals. Those remaining sensitized may be analogous to individuals who become chemically sensitive after long-term, low-dose or single high-dose exposures. The results from HR and LR rats point to

the possibility that FORM-exposure produces a greater dichotomy of responses, a trend which also appears in the locomotor response to a cocaine challenge given at the later withdrawal period. An understanding of the neurochemical alterations within limbic pathways, particularly the nucleus accumbens, may provide insights relevant to behavioral sensitization produced by repeated FORM treatment as well as exposure to other chemicals.

Acknowledgments

The authors would like to thank Debra L. Davidson for technical assistance. This work was supported by the Wallace Genetic Foundation.

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SESSION III
RESPONSES OF SPECIAL HUMAN SUBPOPULATIONS
TO TOXICANTS

Adverse health effects of air pollutants in a nonsmoking population

C. Arden Pope, III

Brigham Young University, 130 FOB, Provo, Utah 84602, USA

Abstract

Utah Valley has provided an interesting and unique opportunity to evaluate the health effects of respirable particulate air pollution (PM_{10}). Residents of this valley are predominantly nonsmoking members of the Church of Jesus Christ of Latter-day Saints (Mormons). The area has moderately high average PM_{10} levels with periods of highly elevated PM_{10} concentrations due to local emissions being trapped in a stagnant air mass near the valley floor during low-level temperature inversion episodes. Due to a labor dispute, there was intermittent operation of the single largest pollution source, an old integrated steel mill. Levels of other common pollutants including sulfur dioxide, ozone, and acidic aerosol are relatively low. Studies specific to Utah Valley have observed that elevated PM_{10} concentrations are associated with: (1) decreased lung function; (2) increased incidence of respiratory symptoms; (3) increased school absenteeism; (4) increased respiratory hospital admissions; and (5) increased mortality, especially respiratory and cardiovascular mortality.

Keywords: Air pollution; Particulate pollution; Respiratory disease

1. Introduction

Utah Valley has provided a useful opportunity to evaluate health effects of respirable particulate air pollution (PM_{10}). The valley is located in Utah County of central Utah. In 1990, the county had a population of about 265 000 of which approximately 188 000 resided in several contiguous cities situated on the valley floor around 1402 m above sea level (Fig. 1). Approximately 90% of the residents of this valley are members of the Church of Jesus Christ of Latter-day Saints (Mormons), which has strong church teachings against smoking. Consequently, only about 6% of the adults smoke.

Utah Valley has moderately high mean PM_{10} levels. During low-level temperature inversion episodes common to winter months, PM_{10} concentrations can become highly elevated due to local emissions being trapped in a stagnant air mass near the valley floor. Regular monitoring of PM_{10} has been conducted at three sites (Fig. 1). Based on data from these three sites, average PM_{10} levels are somewhat uniform throughout the densely populated area of the valley. Also, day-to-day changes in PM_{10} levels across the monitoring sites are highly correlated.

One important consideration of the Utah Valley experience was the intermittent operation of the principal point source of particulate pollu-

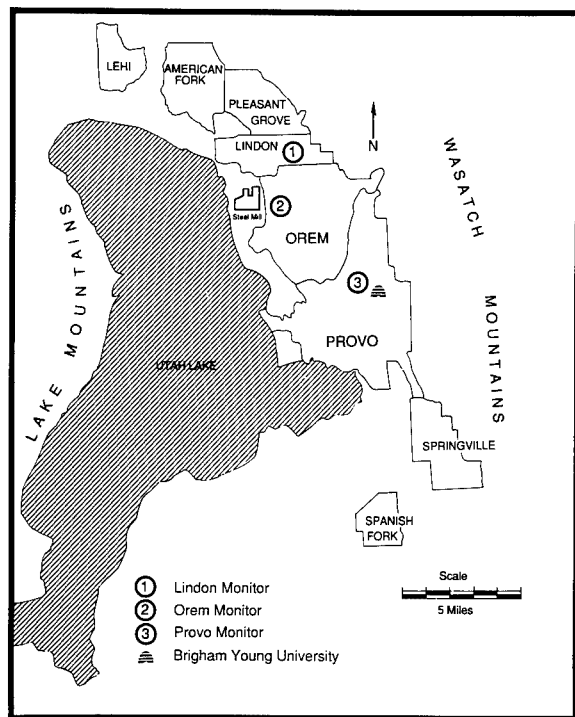


Fig. 1. Map of Utah County in the state of Utah.

tion. This source was an integrated steel mill, built during World War II, and located near the shore of a large freshwater lake on the west side of the populated corridor (Fig. 1). Due to a labor dispute and subsequent change in ownership, the mill was shut down for a 13-month period from 1 August 1986 to 1 September 1987, resulting in a substantial corresponding reduction in local particulate air pollution levels. Another important aspect of Utah Valley is that concentrations of sulfur dioxide (SO_2), ozone (O_3), and acidic aerosols are relatively low or are not strongly positively correlated with particle pollution (Pope, 1995).

There have been several recent studies specific to Utah Valley that have evaluated the health effects of particulate air pollution. An objective of this paper was to contribute to the Conference on Risk Assessment Issues for Sensitive Human Populations. It is not clear that this specific population is relatively more sensitive to the

effects of air pollution than other populations. However, the largely nonsmoking population and the unique research opportunities that have been provided in this study population, have resulted in various relevant studies. This paper will briefly review these studies by focusing on the various health endpoints outlined in Table 1.

2. Summary of observed health effects

2.1. Lung function

Peak expiratory flow (PEF) measures have been widely used as a simple, inexpensive indicator of acute changes in lung function in asthmatics. Daily PEF measurements were gathered in two separate panel studies in the winters of 1989–90 and 1990–91 (Pope et al., 1991; Pope and Dockery, 1992). In these studies there was a total of four panels including: (1) a patient-based panel that consisted of individuals who were receiving medical treatment for asthma and who were referred to the study by local physicians; (2) a panel of “mildly asthmatic” school-aged children that, based on questionnaire information, had ever sneezed without a cold, wheezed for 3 days or more out of the week for a month or longer, and/or had a doctor say the ‘child had asthma’; (3) a panel of symptomatic but relatively healthy school aged children; and (4) a panel of asymptomatic healthy school-aged children.

These studies observed that elevated PM_{10} levels were associated with small but statistically significant reductions in lung function as measured by PEF. For both studies, and for all four panels, there appeared to be associations for up to 5 days of prior PM_{10} exposure. Stronger associations were found when lag structures that included up to 5 days of exposure were included in the statistical models. A $10\text{-}\mu\text{g}/\text{m}^3$ increase in PM_{10} over a period of several days was associated with an average decrease in PEF equal to approximately 0.1 to 0.5%. Although negative associations between PEF and PM_{10} were observed for all four panels, the strength of the association was different across the panels. Relatively weaker associations were found with the sample of asthmatic patients. Asthmatics may

Table 1
Summary of published studies on health effects of particulate air pollution in Utah Valley and neighboring communities

Health endpoints	Study designs	Summary of results
Lung function (Pope et al. 1991; Pope and Dockery 1992)	Panel-based daily time-series	Elevated PM ₁₀ levels were associated with small, transient, but statistically significant declines in lung function (PEF). Such associations existed for asthmatic and non-asthmatic children, and adult smokers with mild to moderate COPD.
Respiratory symptoms (Pope et al. 1991; Pope and Dockery 1992)	Panel-based daily time-series	Strong, statistically significant associations between PM ₁₀ and respiratory symptoms, especially lower respiratory symptoms and cough, were observed.
School absences (Ransom and Pope 1992)	School-based daily and weekly time-series	Grade school absenteeism was significantly higher during and following episodes of elevated PM ₁₀ levels.
Hospitalizations (Pope 1989; Pope 1991)	Population-based monthly time-series	Strong statistically significant associations between the operation of primary particle pollution source (the steel mill), PM ₁₀ pollution, and respiratory admissions were observed.
Mortality (Lyon et al., 1981, 1995; Archer, 1990; Pope et al., 1992; Blindauer et al. 1993)	Population-based daily time-series, case- control, cross- sectional and long-term longitudinal	Statistically significant and consistent associations between PM ₁₀ pollution and total mortality and respiratory and cardiovascular disease mortality were observed. Reported increases in lung cancer risk associated with the pollution have not been as consistent across studies.

not be less susceptible to PM₁₀ pollution but may be controlling their condition through medication. In fact, for the asthmatic panel, the increased bronchodilator usage was strongly associated with PM₁₀ levels. In general, the strongest associations between PEF and PM₁₀ were with the panels of symptomatic but relatively healthy children.

2.2. Respiratory symptoms

In both of the previously mentioned studies that monitored daily PEF levels (Pope et al., 1991; Pope and Dockery, 1992), participants also recorded the presence of specific respiratory symptoms in daily dairies. The use of daily dairies to record respiratory symptoms has proven to be an inexpensive method of evaluating acute changes in respiratory health status. For the patient-based asthmatic panel, the PM₁₀ levels were not significantly associated with respiratory symptoms, but were strongly associated with extra asthma medication use. However, for all three of the school-based panels, strong, statisti-

cally significant associations between PM₁₀ and respiratory symptoms were observed. These associations were especially strong for lower respiratory symptoms and cough.

As with the association with PEF, there appeared to be a lag structure between exposure and respiratory symptoms. Associations for up to 5 days of prior PM₁₀ exposure were observed. Stronger associations were found when lag structures that included up to 5 days of exposure were included in the statistical models. A 10- $\mu\text{g}/\text{m}^3$ increase in PM₁₀ over a period of several days was associated with an average increase in lower respiratory symptoms and/or cough equal to approximately 2–7%. As with the PEF results, the respiratory symptom results indicate that both symptomatic and asymptomatic children may suffer acute health effects of respirable particulate pollution.

2.3. School absences

A study that assessed the association between PM₁₀ pollution and elementary school absences

in Utah Valley has also been conducted (Ransom and Pope, 1992). This study compiled school absenteeism and PM_{10} data for the six school years of 1985–1990. Weekly absenteeism data from a full school district and daily data from a single elementary school were analyzed for grades kindergarten through 6th. For both data sets, regression models were estimated. For the weekly school district data, absenteeism was regressed on PM_{10} pollution levels, weather variables, and variables indicating month of school year, and holiday weeks. For the daily elementary school data, similar models were estimated. However, variables that indicated day-of-week, and days preceding and following holidays could also be included in the models. Many things, including weather, day-of-week, holidays, and month of year influenced absenteeism.

Estimated associations between absenteeism and PM_{10} pollution in both data sets were positive and statistically significant. PM_{10} effects persisted for up to 3 or 4 weeks. Regression results from both data sets indicated that an increase in 28-day moving average PM_{10} equal to $100 \mu\text{g}/\text{m}^3$ was associated with an increase in the absence rate equal to approximately two percentage points, or an increase in overall absences equal to approximately 40%. Similar relationships were observed for all grade levels, although the response of absences to air pollution was generally greater for grades 1–3 compared with grades 4–6. These associations were robust to changes in model specification.

2.4. Hospitalizations

Two studies have explored the association between PM_{10} pollution and respiratory hospitalizations in Utah Valley (Pope, 1989, 1991). Both studies utilized the natural experiment that was provided due to the local steel mill being closed for 13 months. In the first study, monthly hospital admission data were collected from April 1985 through February 1988, from all four hospitals in the county. Using diagnosis-related groups (DRG) coding, this study focused on hospital admissions for bronchitis, asthma, pneumonia and pleurisy. PM_{10} pollution levels were observed to be strongly associated with respiratory

hospital admissions. During months when the 24-h PM_{10} levels exceeded $150 \mu\text{g}/\text{m}^3$, average admissions for children nearly tripled and in adults the increase was 44%. During the winter months when the steel mill was operating, versus the winter months when it was not, PM_{10} levels were nearly double and children's admissions for these respiratory diseases were two to three times higher. Formal multiple regression analysis also revealed that PM_{10} levels were strongly correlated with hospital admissions. They were more strongly correlated with children's admissions than with adult admissions and more strongly correlated with admissions for bronchitis and asthma than with admissions for pneumonia and pleurisy.

One important concern about the first hospitalization study was that increases in contagious illnesses such as influenza or respiratory syncytial virus (RSV) occurred coincidentally during winters when the steel mill was open but not when it was closed (Lamm et al., 1991). Therefore, the second study was conducted (Pope, 1991). This study expanded the analysis by including data from neighboring Salt Lake and Cache valleys and by extending the study period. This second study also used more detailed coding on diagnoses (including ICD-9 codes) and ages. This study observed that bronchitis and asthma admissions for preschool-age children were approximately twice as frequent in Utah Valley when the steel mill was operating versus when it was not. Similar differences were not observed in the neighboring valleys. Various other analysis, including regression analysis, demonstrated a statistical association between respiratory hospital admissions and PM_{10} pollution but did not indicate that the pollution-hospitalization association was due to coincidental epidemics of influenza or RSV. Based on these studies, it is estimated that a $10\text{-}\mu\text{g}/\text{m}^3$ increase in PM_{10} over a period of a month or more is associated with an increase in respiratory hospitalizations equal to approximately 3–7%.

2.5. Mortality

There have been several air pollution mortality studies conducted in Utah Valley. The first of

these studies evaluated the spatial distribution of lung cancer cases in Utah County between 1966 and 1975 (Lyon et al., 1981). Lung cancer cases were clustered in relation to the distance from the steel mill coke ovens. Up to a distance of 5 miles, an excess of lung cancer cases over expected number of cases was observed. For up to 3 miles the relative risk of lung cancer was 44% higher. This study suggested that the risks of dying of lung cancer are highest for those living closest to coke ovens. However, the association was not "statistically significant" and, although the smoking rates are on average very low, cigarette smoking was not precluded as a confounding factor since it was not controlled for in this study.

In a second study (Archer, 1990), it is noted that because the exposure to pollution is fairly uniform across the populated area of Utah Valley, the methodology used in the previous lung cancer study was probably unable to observe most of the impact of air pollution. This study compared age-adjusted death rates for malignant and nonmalignant respiratory disease across Utah, Salt Lake, and Cache Counties. Utah and Cache Counties had very similar demographics and very low smoking rates. They also had nearly identical mortality rates due to malignant respiratory disease until about 10 years after the steel mill was constructed in Utah county causing substantial increase in pollution. Subsequent differences in mortality rates from both malignant and nonmalignant respiratory disease were pronounced and statistically significant. Based on changes in both spatial and longitudinal differences in death rates, it was estimated that 30–40% of respiratory disease deaths, or approximately 5% of all deaths, were associated with the air pollution in Utah County. Higher levels of cigarette smoking in Salt Lake County made it impossible to separate out the pollution effect, but the results suggested that pollution also had a substantial health effect on Salt Lake County residents. Another paper (Blindauer et al., 1993) does not address the observed nonmalignant mortality association, but suggests that the apparent lung cancer effect is not consistent with alternative approaches to controlling for smoking.

A daily time-series mortality study has also been conducted that analyzed daily deaths in Utah County for a nearly 5-year period (Pope et al., 1992). Daily death counts ranged from 0–12. Mean daily deaths equaled 2.7 deaths per day. When daily death counts were averaged over days with differing pollution levels, it was observed that for every increase in PM_{10} levels of approximately $50 \mu g/m^3$ there was an increase in average mortality equal to several percent. This simple univariate analysis however did not account for the potentially confounding effects of weather. Poisson regression analysis was used to regress daily death counts on PM_{10} pollution levels, controlling for weather variability. A significant positive association between non-accidental mortality and PM_{10} pollution was observed. The strongest association was with 5-day moving average PM_{10} levels. An increase in 5-day moving average PM_{10} levels equal to $100 \mu g/m^3$ was associated with an estimated increase in deaths/day equal to 16%. The association with mortality and PM_{10} was largest for respiratory disease deaths, next largest for cardiovascular deaths, and smallest for all other deaths.

Recently, the results of a similar study have been reported (Lyon et al., 1995). In this study when PM_{10} was treated as a continuous variable in the regression model as was done in the earlier paper, nearly identical results were obtained. This study, however, conducted most of its analysis with a dichotomous pollution variable equal to 1 if PM_{10} is greater than $50 \mu g/m^3$ and 0 otherwise. The data were also divided-up by year, season, three places of death categories, six age group categories, in addition to three cause-of-death categories. Such data stratification often resulted in regressions using extremely small counts. Not surprisingly, the use of the dichotomized pollution variable and the excessive stratification of the data often resulted in substantially weaker statistical results that are extremely difficult to interpret. The authors suggested a non-causal interpretation. Nonetheless, pooled analysis showed positive associations between PM_{10} and mortality that were strongest with cardiopulmonary disease mortality. Also, when the age groups

were pooled, it was observed that the PM_{10} -mortality association was strongest for those that died in the home and weakest for those that died while in the hospital. Such results are consistent with the interpretation that particulate pollution is a risk factor for cardiopulmonary disease mortality.

3. Discussion

Air pollution studies in Utah Valley are of interest, not because of their anomalous results. The results are largely consistent with those observed in other study areas as has been summarized in several more general reviews of particulate pollution and health (Ostro, 1993; Lipfert, 1994; Dockery and Pope, 1994; Schwartz, 1994; Pope et al., 1995). The Utah Valley studies are of interest because of the low smoking rates of the population, the unique natural experiment afforded by the intermittent operation of the steel mill, the low levels of various co-pollutants, and the availability of relatively reliable pollution and health endpoint data. The Utah Valley studies are also of interest because of the broad range of health endpoints that have been studied, because the overall results are largely consistent and coherent, and because most of the study results have been published and have been and are being subjected to critical review.

Because these studies looked at various health endpoints under differing conditions and constraints, they necessarily used a variety of study designs and differing data analytic techniques. Each of these individual studies had limitations imposed upon them by data and analytic constraints. There are important concerns pertaining to these studies that reflect legitimate skepticism about inherent limitations imposed upon epidemiologic studies. For example, there are issues related to methodologic or analytic bias and concerns about confounding. A recent review of the Utah Valley studies focused on these concerns (Pope, 1995). It concluded that given currently available information, it is improbable that the apparent air pollution-related health effects were due to methodologic bias or systematic residual confounding of cigarette smoking, socio-

economic factors, epidemics of contagious illnesses, or weather. It is also unlikely that the results are due to confounding by sulfur dioxide, ozone, or aerosol acidity.

Another set of concerns regarding these epidemiologic studies relates to biological significance or plausibility of the observed associations (Utell and Samet, 1993). These studies provide little information on the specific biological mechanisms responsible for the observed effects or on the toxicity of specific constituents of particulate pollution. Biological plausibility, however, is enhanced by the observation of a variety of cardiopulmonary health effects and by the fact that non-cardiopulmonary health endpoints were not typically associated with particulate pollution. Bates (1992) has pointed out that perhaps the most intriguing observation from these studies is the coherent cascade of cardiopulmonary health effects. These studies, taken as a whole, provide evidence that respirable particulate pollution, some constituent of this pollution, or some pollutant that is closely associated with respirable particulate pollution, is an important risk factor for respiratory disease and cardiopulmonary mortality.

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Comparison of four human studies of perinatal exposure to methylmercury for use in risk assessment

John L. Cicmanec

*National Risk Management Research Laboratory, U.S. Environmental Protection Agency,
26 West M.L. King Drive, Cincinnati, OH 45268, USA*

Abstract

Newer data from human epidemiologic studies of methylmercury (MeHg) poisoning in which perinatal exposure occurred are available from four distinct populations. The results of an Iraqi grain-consuming population are compared to results from studies performed in fish-consuming groups in the Faroe Islands, the Seychelles Islands and in Peruvian fishing villages. A comparison of the results indicate that the Iraqi population does not represent a sensitive subpopulation within a perinatal group, but rather the relative lower threshold identified from this study was the result of confounders. Use of this benchmark dose for regulatory purposes may place a severe limitations upon fish consumption in the United States that is not fully supported by the scientific data.

Keywords: Methylmercury; Perinatal exposure; Comparative human studies; Risk assessment

1. Introduction

Quantitative noncancer risk assessment within the Environmental Protection Agency is performed by establishing a reference dose for individual chemical pollutants. A reference dose is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily dose for humans (including sensitive subpopulations) that is likely to be without deleterious effects throughout a lifetime. Normally a reference dose is derived from results of animal toxicity studies in which a NOAEL (No observed adverse effect level) and LOAEL (Lowest observed adverse effect level) are taken from the dose levels used in the animal studies and serve to define the toxicity threshold

for that particular compound. In many instances a complete base of experimental data is not available (such as the lack of reproductive or developmental studies) to precisely define the most sensitive toxicity threshold in which case uncertainty factors are applied to the NOAEL that the existing data can establish.

The application of a safety factor to allow for sensitive subpopulations among humans is historically one of the two original areas for uncertainty in risk assessment. In an original paper on risk assessment (Lehman and Fitzhugh, 1954) a 10-fold safety factor was applied for sensitive subpopulations among humans and another 10-fold factor was used to adjust for extrapolation of data from animal studies to humans.

With minor adjustments, a similar system is used to determine a toxicity threshold from human epidemiologic studies. However, particular problems arise regarding quantitation of daily doses and identifying dose groups.

When human data serves as the primary basis for a risk assessment, such as is the case for MeHg, then the principles for applying uncertainty factors are modified and the topic of sensitive subpopulations becomes even more critical.

2. Background

In 1971-1972 many citizens throughout rural Iraq were exposed to methylmercury-treated seed grain that was mistakenly used in home-baked bread. Latent neurologic toxicity was observed in many adults and children who had consumed bread over a 3-month period. A retrospective study of the affected population was conducted by University of Rochester and Iraqi physicians (Marsh et al., 1987) and it was determined that infants born to mothers who ate contaminated bread during gestation exhibited more serious neurologic signs than their mothers or the general adult population. Often infants exhibited neurologic abnormalities while their mothers showed no signs of toxicity. Among the signs noted in the infants exposed during fetal development were cerebral palsy, altered muscle tone and deep tendon reflexes as well as delayed developmental milestones, i.e. walking by 18 months and talking by 24 months. The neurologic signs noted in adults included paresthesia,

ataxia, reduced visual fields and hearing impairment. Some mothers experienced paresthesia and other sensory disturbances but these symptoms were not necessarily correlated with neurologic effects in their children. Unique analytic features of mercury, that is, analysis of segments of hair correlated to specific time periods in the past permitted approximation of maternal blood levels that the fetuses were exposed to in utero (Table 1). The data collected by Marsh et al. (1987) summarized clinical neurologic signs of 81 mother-child pairs. From X-ray fluorescent spectrometric analysis of selected regions of maternal scalp hair, concentrations ranging from 1 to 674 ppm were determined, then correlated with clinical signs observed in the affected members of the mother-child pairs. Among the exposed population there were affected and unaffected individuals throughout the dose-exposure range. Based upon this data, the U.S. Environmental Protection Agency established a Reference Dose for MeHg of 0.0001 mg/kg-day. This value was determined by modelling the entire dose range using those infants that showed signs and creating arbitrary dose groups. Since all data points were identified by the mean hair concentration for mercury of the mother during gestation, using a polynomial model the 10% incidence rate above background levels was found to be 11 ppm. By using appropriate conversion factors the dose level of 0.0001 mg/kg-day was determined.

Three subsequent studies in high fish-consuming populations were made which do not support the results of the Iraqi study. Neither delayed

Table 1
Perinatal exposure to methylmercury: comparison of study parameters for mother-child pairs of four studies

	Iraq	Seychelles	Faroe	Peruvian
Population and subgroup studied	Grain-consuming	Fish-consuming	Fish-consuming	Fish-consuming
Number of subjects	81	789	442	342
Range of hair Hg conc.	0-148 ppm	0.6-18 ppm	0-10 ppm	1.2-18 ppm
Maternal intake	0-1801 $\mu\text{g}/\text{day}$	6-106 $\mu\text{g}/\text{day}$	0-112 $\mu\text{g}/\text{day}$	3.3-78 $\mu\text{g}/\text{day}$

For the Iraqi study, malnutrition and severe parasitism of the test subjects were significant confounders as was lack of accurate information regarding the children's ages. For the Faroe Island study, alcohol consumption and PCB exposure appear to be significant confounders.

onset of walking nor delayed onset of talking occurred among nearly 1500 children that had been exposed perinatally to similar doses of MeHg. A study (Marsh et al., 1994) of fetal exposure to MeHg through maternal consumption of fish was completed in the Peruvian fishing village of Mancora in 1992. Hair samples were obtained from 369 pregnant women and neurologic examinations were performed on 194 of the children from these pregnancies. Of this cohort, there were 131 mother-infant pairs with complete clinical data and hair samples that provided quantitative measures of MeHg exposure during pregnancy. The mean hair MeHg levels ranged from 1.2 to 30 ppm with a mean of 8.3 ppm. The profiles of mean and peak MeHg levels showed little variation and the mothers had been consuming MeHg-contaminated fish for many months prior to the births of their children. This information indicates that steady state conditions for MeHg had been attained. The authors broke the study population into three dose groups of 0-5 ppm, 5-9 ppm and 9-28 ppm. Thorough neurologic examinations of the children including analysis for general psychomotor retardation did not reveal any abnormalities. The study protocol included examinations for maternal paresthesia, speech retardation, muscle tone evaluation, determination of primitive and tendon reflexes, ataxia, and mental and motor retardation. Based on the results of this study, a free-standing NOEL of 18 ppm in maternal hair was established.

The term free-standing NOEL is used when no adverse effects are noted in the highest dose group. Data from such a study is limited because a toxicologic threshold is not determined and the critical adverse effect is not defined. The value of such studies for risk assessment is dependent upon the thoroughness of study design and upon the extent of various end points that are measured in the test subjects. When such studies are of very thorough design, the test results they provide can be used to refute studies of lesser quality that establish lower thresholds.

In 1986, the University of Rochester team in conjunction with the Seychelles Island government initiated a large study in which the effects

of low level MeHg exposure on fetal development was examined (Cox et al., 1994; Davidson et al., 1994; Myers et al., 1994). Among the Seychelles Island population, fish are plentiful and are the primary dietary source of protein. A pilot study involving 789 mother-infant pairs was initiated in which maternal hair samples and umbilical cord blood were measured for MeHg content using atomic absorption spectroscopy. Multiple hair samples were collected during gestation with MeHg levels ranging from 0.6 to 36 ppm with a median of 6.6 ppm (Table 1). These values correlated with daily maternal ingestion rates of 6-106 $\mu\text{g}/\text{day}$. The endpoints evaluation during the pilot study included a general neurologic evaluation and the Denver Development Screening Test (DDST) in addition to thorough examinations of physical development. The precise age of each child was known and testing was performed on each child at 6 months of age. In addition to DDST testing, a medical history was obtained and a neurologic examination, the Fagan Test and collection of breast milk and hair from both the mother and infant was performed. The results of developmental tests including the DDST as well as tests for limb tone and deep tendon reflexes revealed that there were essentially no differences among any of the four separate dose groups.

Statistical testing of the results of the DDST results was made for the following co-variables: sex, Apgar score, age at testing, maternal age, birth weight, cigarette and alcohol consumption by mothers, and neonatal complications. None was found to have significant influence. After the pilot study was completed, the test battery was expanded for testing at 19 and 29 months of age. The Bayley Scale of Infant Development (BSID) was chosen as the primary test, in addition, expanded end-points that were included were visual recognition memory, cognitive tests, educational tests, and language tests. The expanded co-variables that were added included socio-economic factors, parental education, post natal exposure, and language at home. The investigators felt that the BSID provided the most significant results. BSID scores were normally distributed and the mean Mental Scale Index at both 19 and

29 months was comparable to performance by US children. Multiple linear regressions using BSID mean scores were used to evaluate effects of MeHg. The results showed no effect of MeHg on BSID scores at either age. For the Seychelles island children, the mean Psychomotor Scales Indices at both ages were 2 standard deviations higher than US norms but consistent with previous findings of motoric precocity for children following early experience in outdoor settings. Based on these findings, a free-standing NOAEL of 15 ppm in maternal hair is established.

A comprehensive study was undertaken in the Faroe Islands in 1986 in which the effects of MeHg and PCB exposure on human neonates was investigated (Grandjean et al., 1994). Evaluation of the possible neurologic effects were made using standardized neurologic and developmental test procedures when the children were 2, 5, and 7 years of age. A group of nearly 1000 children were evaluated. Tests of motor and cognitive function were made when the children reached 5 and 7 years of age. In addition to the DDST, tests for motor development included the Neurobehavioral Evaluation System (NES) finger tapping test and the NES Hand-Eye Coordination test; the test for language skills was the Boston Naming Test; the test for visuospatial skills were the WISC-r Block Designs and Bender Gestalt test; and the tests for memory were the Tactual Performance Test and California Verbal Learning Test. The investigators placed the children into four study groups based on maternal hair concentrations of 2.0 ppm, 3.9 ppm, 4.5 ppm and 8.1 ppm. The authors indicated that Year 1 data suggest that some neurobehavioral dysfunction is related to maternal seafood intake during pregnancy, particularly on WISC-R digital spans forward and the Boston Naming Test. Although the medians for the test scores are similar or identical across the study groups, the upper exposure groups had many more instances of scores in the lowest quartile. In addition to these results, positive findings were seen on the NES continuous performance test. The standard deviation obtained for reaction time and the number of false positive errors were higher than expected. However, these findings

must be viewed with caution. The authors state that these data cannot be directly related to mercury exposure until further investigation of potential confounders including exposure to PCBs has been concluded. In a more recent published report (Grandjean et al., 1995), a high correlation between elevated levels of MeHg in maternal hair was associated with an earlier age of reaching the developmental milestones of onset of sitting, creeping and rising for a group of 583 infants that were breastfed in the Faroe Islands. While this finding may be counterintuitive, the authors emphasize that this finding is likely the result of the benefits of breastfeeding and the potential toxic effects of MeHg did not override these benefits.

3. Discussion

The preliminary results reported for the Seychelles Islands, the Faroe Islands and the Peruvian fishing villages stand in stark contrast to the results for the Iraqi study (Table 1). Although all four studies are concerned primarily with populations that described perinatal exposures ranging from 0 to 40 ppm in maternal hair (or maternal dietary intake of 0 to 260 $\mu\text{g}/\text{day}$), only the Iraqi study demonstrated overt neurologic effects in this dose range. However, the validity of these effects have been questioned by members of the toxicology community including the study authors.

The basic point to be considered is: was the Iraqi population unusually sensitive to the effects of MeHg or was some other factor or factors at play? Concerns raised by the primary investigators, Drs. Clarkson, Cox, and Myers (Moskowitz et al., 1994) indicate that the latter choice is more likely. The clinical neurologic examinations of the infants that had been exposed perinatally to MeHg were performed by Dr. Myers and Dr. Marsh in rural Iraq. One of the primary concerns they expressed is developmental standards based in Western Cultures of walking by 18 months and talking by 24 months of age may be unrealistic for the agrarian Moslem cultures of Iraq since the mothers do not talk to their infants as

is done in Western cultures. In addition, since much of the population in rural Iraq is nomadic, infants are not encouraged to walk at an early age. Use of such developmental standards is not appropriate for the Iraqi population. Misapplication of such a standard is not the same as recognizing a sensitive subpopulation among infants that were exposed perinatally. In addition, knowing the precise age of children is not a matter of high importance in the Moslem culture. The mothers could only approximate their children's ages relative to religion holidays. They did not know their ages precisely. Another area for concern is whether or not intercurrent disease may have contributed to the adverse neurologic manifestations that were observed in the Iraqi population. Again the study authors have reported that heavy parasitism and borderline malnutrition were commonplace among the Iraqis that were the subjects of their study. In view of the compromises to health that these conditions present makes the interpretation of study results particularly tenuous. The choice of the Marsh study as the basis for noncancer risk assessment of MeHg is compromised in view of these limitations and further emphasizes that this population did not represent a sensitive subpopulation.

The difference in results could be attributed to the grain-consuming practices of the Iraqi population in contrast to primarily fish consumption in the three other studies. To a large extent this point is moot since likely human exposure to MeHg will be from the consumption of high levels of fish; only accidental poisonings by consumption of contaminated seed-grain such as occurred in Iraq is the only scenario aside from fish consumption that is likely to occur.

A subpopulation may be deemed "sensitive" if members react more strongly to a given concentration of the agent in question than does the population at large. For a multifactorial endpoint (such as all the mild neurological symptoms involved with MeHg), this is not the same as a subpopulation with increased prevalence of the endpoint. This is an important distinction with respect to a situation like asthmatics and respiratory irritants, for example, for which prevalence of the symptom indicates sensitivity. All

persons with CNS signs, low IQ or paresthesia are not necessarily more sensitive to MeHg and thus there may be no way to identify "sensitives" a priori. However, there appears to be sufficient evidence to conclude that the developing fetus constitutes a bona fide class of sensitives, regardless of the parent population. The only way sensitivity can be determined is by comparing the dose-response functions assuming that these dose-response functions are free of the effects of confounding variables. Since this group of studies have involved human exposures, the dose-response slope has been delineated using modelling procedures such as hockey-stick functions which can only crudely define a threshold.

While the results of this group of studies has proven to be an interesting challenge in risk assessment, the ultimate conclusion is that only fish-consuming populations in which steady-state conditions of tissue MeHg concentration exist should be used. Results of outlying studies such as the Iraqi study are aberrations.

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Toxicology 111 (1996) 163–178

TOXICOLOGY

Role of nutrition in the survival after hepatotoxic injury

Sanjay Chanda, Harihara M. Mehendale*

Division of Toxicology, College of Pharmacy and Health Sciences, Northeast Louisiana University, Monroe, LA 71209-0470, USA

Abstract

Nutritional status is an important factor in determining susceptibility to toxic chemicals. While macro and micronutrients may affect many aspects of Stage I and Stage II of toxicity, in this paper, the influence of macronutrients as sources of energy required for cell division and tissue repair mechanisms on the outcome of hepatic injury is discussed. Male Sprague–Dawley rats maintained on normal rodent chow and 15% glucose (as a source of energy for the centrilobular hepatocytes) in drinking water for 7 days experienced an increased lethality from structurally and mechanistically different centrilobular hepatotoxicants (acetaminophen, thioacetamide, chloroform and carbon tetrachloride), while male Sprague–Dawley (S-D) rats fed rat chow containing palmitic acid (PA, 8% w/w, as a source of energy for the periportal hepatocytes) and L-carnitine (LC, 2 mg/ml, as a mitochondrial carrier for the supplemented fatty acids) in drinking water for 7 days were protected from a LD_{100} dose (600 mg/kg, i.p.) of thioacetamide (TA). Indices of cell division revealed that cell cycle progression in the liver play a very critical role in determining the final outcome of hepatotoxic injury. These results confirmed our hypothesis that cell division and tissue repair play a critical role in survival after life-threatening hepatotoxic injury. Any manipulation directed towards altering a prompt and exacting compensatory cell division and tissue repair responses after hepatotoxic injury would also alter the final outcome of the toxicity. These studies indicate that the source of cellular energy can decisively influence the compensatory response of the target tissue to alter the outcome of hepatotoxic injury. Since nutritional status is known to vary widely among human populations, these could contribute enormously to susceptibility of human populations to toxic chemicals.

Keywords: Cell division; Tissue repair; Thioacetamide; Glucose; Palmitic acid; L-carnitine

1. Introduction

With the possible exception of neuronal tissue, organisms are known to compensate for tissue damage by stimulating cell division intended for replacement of dead cells, thereby restoring tissue

structure and function. In dose-response studies it has been shown that stimulation of cell division and tissue repair are simultaneously occurring biological responses to tissue injury (Rao et al. 1994; Mangipudy et al., 1995a). Survival after hepatocellular injury and necrosis depends on ability of the remaining hepatocytes to regenerate and restore an adequate population of functioning cells (Holecek et al., 1991). Adequate nutritional support plays an important role in facilitating liver regeneration after severe liver

Current address: University of North Carolina at Chapel Hill, Curriculum in Toxicology, U.S. Environmental Protection Agency, Health Effects Research Laboratory, MD-74, Research Triangle Park, NC 27711, USA.

*Corresponding author.

damage (Bengmark et al., 1965; Zivny and Simek, 1989). As a rule, the administration of nutritional substances is evaluated only from their potential to generate energy, without considering whether these substrates also influence the cell division and tissue healing processes either via their effects on the relevant energy metabolism or via other mechanisms. Since marked hypoglycemia follows severe liver damage, glucose is used as a source of energy in clinical situations of liver damage though its contribution to liver regeneration and/or survival is unclear.

There is considerable interest in the ability of the liver to "regenerate" during normal growth and development, during pathophysiological alterations, and during chemical-induced hepatotoxic injury (Columbano et al., 1990). Several studies have established the critical role of hepatocellular regeneration and tissue repair in determining the final outcome of liver injury induced by wide variety of model hepatotoxicants such as chloroform, galactosamine (Mehendale et al., 1989; Abdul-Hussain and Mehendale, 1992), etc. These studies have led to the concept that the critical determinant of the ultimate outcome of chemically induced hepatotoxicity is the extent of sustainable tissue repair occurring after infliction of injury (Calabrese et al., 1993). Furthermore, cell division and hepatic tissue repair stimulated by a protective dose has been shown to be the mechanism underlying CCl_4 and thioacetamide autoprotection (Rao and Mehendale 1991; Mehendale et al., 1994; Thakore and Mehendale, 1994; Mangipudy et al., 1995a).

Hepatocyte proliferation occurs in a unique spatial pattern that is related to the localization of hepatocytes in the different zones of acinus. Studies on the distribution of DNA-synthesizing cells in Rappaport's zones I, II, and III of the liver acinus, carried out at different intervals after liver resection (Gebhardt, 1988) showed that after a toxic insult to the liver, cellular regeneration starts in the periportal region which derives its energy from fatty acid oxidation.

Thioacetamide (TA), originally used as a fungicide, a potent hepatotoxin and carcinogen, has been shown to stimulate hepatic DNA synthesis (Reddy et al., 1969). In recent studies, it has been

shown that this DNA synthesis is directed towards stimulated cell division and tissue repair (Mangipudy et al., 1995a,b). Thioacetamide is oxidized by the mixed function oxidase system to sulfoxide and sulfone metabolites, which bind to liver macromolecules causing toxicity (Hunter et al., 1977).

L-Carnitine (LC) is best known for its role in facilitating entry of long-chain fatty acids into mitochondria for utilization in energy generating processes. Long chain fatty acids cannot enter mitochondria independent of translocation as esters of carnitine (Rebouche, 1992). Therefore, any dietary supplementation of long chain fatty acids intended for augmented mitochondrial oxidative phosphorylation should be accompanied by L-carnitine supplementation as well to ensure mitochondrial delivery of fatty acids.

The mechanisms that regulate proliferation of liver cells is of biomedical interest with direct applications in clinical medicine. Proliferation of liver cells is critical for patient survival in many hepatic diseases. In drug overdosage or poisoning episodes, the present line of therapy aims only at blockade of any additional liver injury from hepatotoxicants or hepatic diseases. While this antidotal therapy might be effective in preventing additional mechanism-driven injury, patient survival depends heavily on the ability of the remaining hepatocytes to divide in order to replenish the dead or dying cells. Death usually occurs when the regenerating ability of the liver is compromised. If liver cell division can be stimulated after massive hepatic damage by some therapeutically compatible mechanism, then it might be possible to prevent death from even massive hepatotoxic injury.

2. Materials and methods

2.1. Chemicals/reagents

All of the chemicals used for the Northern blot analyses were gifts from The Chemical Industry Institute of Toxicology (CIIT, RTP, NC). Monoclonal primary antibody to proliferating cell nuclear antigen (PCNA) was purchased from Dako (Carpinteria, CA). Radioimmunoassay kits

for determination of insulin, glucagon and C-peptide were purchased from Diagnostic Products Corporation (Los Angeles, CA). All other chemicals, unless specified otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Animals and housing environment

Adult male Sprague–Dawley (Harlan Sprague–Dawley Inc., Madison, WI) rats (200–250 g) were used for all the experiments. The rats were housed in polycarbonate shoe-box style cages over sawdust bedding known to be free of any chemical contaminants. Rats were maintained in a temperature ($21 \pm 1^\circ\text{C}$) and humidity (50–80%) controlled environment with a 12-h light/dark cycle (lights on at 0700 h) in our central animal facility. Rats were allowed 7 days to get accustomed to the environment before use in the experiments.

The optimum concentration of glucose that can be used without adversely affecting food consumption (15% glucose in drinking water) was established by preliminary studies. Four structurally and mechanistically differing model hepatotoxicants were used for this study. Rats were divided into several groups as shown in Table 1. On day 8 hepatotoxicants were admin-

istered as indicated in Table 1. The grouping of the rats for the experiments with palmitic acid (PA) and L-carnitine (LC) is shown in Table 2.

2.3. Biochemical assays

Plasma enzyme and glucose levels were determined by commercially available kits (#59 UV for ALT and #510 for glucose from Sigma). Hepatic glutathione (GSH) content was measured by following the method of Reed et al. (1980). Plasma insulin, C-peptide and glucagon levels were determined using the kits made by Diagnostic Product Corporation (#TKIN1, KPED1 and KGND1 for insulin, C-peptide and glucagon, respectively). Fatty acid and carnitine concentrations in plasma and liver were determined by the method of Demacker et al. (1982) and of Nakano et al. (1989), respectively.

2.4. Proliferating cell nuclear antigen (PCNA) immunohistochemical staining

Livers isolated from rats were cut into small pieces in 10% neutral buffered formalin and fixed in the same fixative for 72 h. After 72 h they were transferred to 70% alcohol until they were processed further. The method followed for PCNA was described by Greenwell et al. (1991).

Table 1

Effect of glucose loading on the toxicity of acetaminophen, thioacetamide, chloroform and carbon tetrachloride in male Sprague–Dawley rats

Group	N	Treatment	Hepatotoxicant	% Lethality
I	10	Glucose (15%) in drinking water	Acetaminophen	70
II	10	Glucose (15%) in drinking water	Thioacetamide	100
III	10	Glucose (15%) in drinking water	Chloroform	100
IV	10	Glucose (15%) in drinking water	Carbon tetrachloride	100
V	10	Regular drinking water	Acetaminophen	20
VI	10	Regular drinking water	Thioacetamide	0
VII	10	Regular drinking water	Chloroform	50
VIII	10	Regular drinking water	Carbon tetrachloride	30

Rats of all the groups received normal rat chow ad libitum during the entire period of study. Rats of the first four groups (I–IV) received 15% glucose in drinking water during the entire period of the study. The rest of the groups received regular tap water. On day 8 of glucose loading rats of groups I and V were injected with acetaminophen (800 mg/kg, i.p.), groups II and VI received thioacetamide (300 mg/kg, i.p.), groups III and VII received chloroform (0.3 ml/kg, i.p.) in corn oil and groups IV and VIII received carbon tetrachloride (1.9 ml/kg, i.p.) in corn oil. The rats were observed twice daily for 14 days after the last treatment.

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Table 2

Influence of nutritional supplementation with palmitic acid and L-carnitine on thioacetamide lethality in male Sprague-Dawley rats

Groups	Diet	% Lethality
I	Palmitic acid + L-carnitine	0
II	Normal diet + tap water	100
III	Palmitic acid + tap water	90
IV	Normal diet + L-carnitine	90

Each group had 10 rats. Palmitic acid (8%, w/w) was included in the powdered rat chow for groups I and III and L-carnitine (2 mg/ml) was added to the drinking water for groups I and IV. The rats were maintained on their respective diets throughout the entire period of study. On day 8 they received thioacetamide (600 mg/kg, i.p.). The rats were observed twice daily and mortalities were recorded for 14 days after the TA treatment. First lethality occurred 4 days after the TA treatment (group II) and the last lethality was observed 7 days after TA treatment (groups III and IV).

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2.5. RNA extraction and Northern blot hybridization

Samples of livers were used for RNA extractions employing a modification of the procedure by Chomczynski and Sacchi (1986). The RNA was denatured in formaldehyde/formamide, fractionated by electrophoresis, and then transferred to nitrocellulose (Thomas, 1980).

2.6. Statistical analysis

The means \pm S.E.M. were calculated for all values. Statistical differences between two groups (control and treated) were determined by the Student's paired *t*-test, while the difference between the groups at different time points were found by Generalized Regression Model (GLM) followed by Fisher's Least Significant Difference (LSD) using Statistical Analysis Software (SAS).

3. Results

3.1. Effect of glucose loading

The preliminary studies established that the doses of different hepatotoxicants used for the study were either non-lethal or only caused mar-

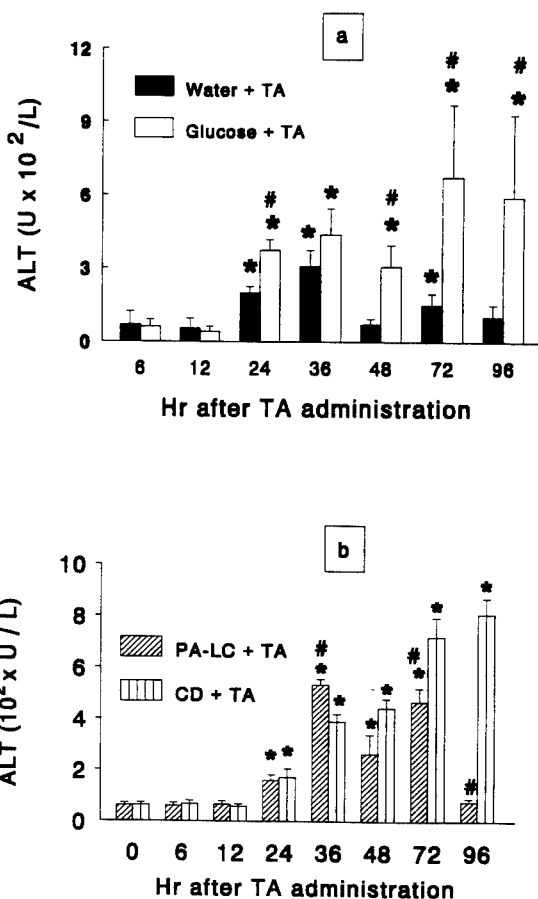


Fig. 1. (a) Plasma alanine aminotransferase (ALT) levels during a time-course after thioacetamide administration in glucose-loaded rats. Details of treatment are in Table 1. (b) Plasma alanine aminotransferase (ALT) levels during a time-course after thioacetamide administration in PA-LC supplemented rats and the rats on normal diet (CD). Details of treatment are in Table 2. Groups of 4 rats were used for serum enzyme measurements at each time point for each group. *Significantly different from control ($P \leq 0.05$). #Significantly different from the group receiving tap water and normal diet ($P \leq 0.05$). Fig. 1a and b have been adopted from Chanda and Mehendale (1994,1995) by permission of FASEB J.

ginal lethality. All hepatotoxicants under investigation became highly lethal in glucose-loaded rats. The glucose-loaded groups receiving a marginally lethal dose (20% lethality) of acetaminophen (Table 1, groups I and V) experienced 70% lethality. All rats receiving a non-lethal dose of

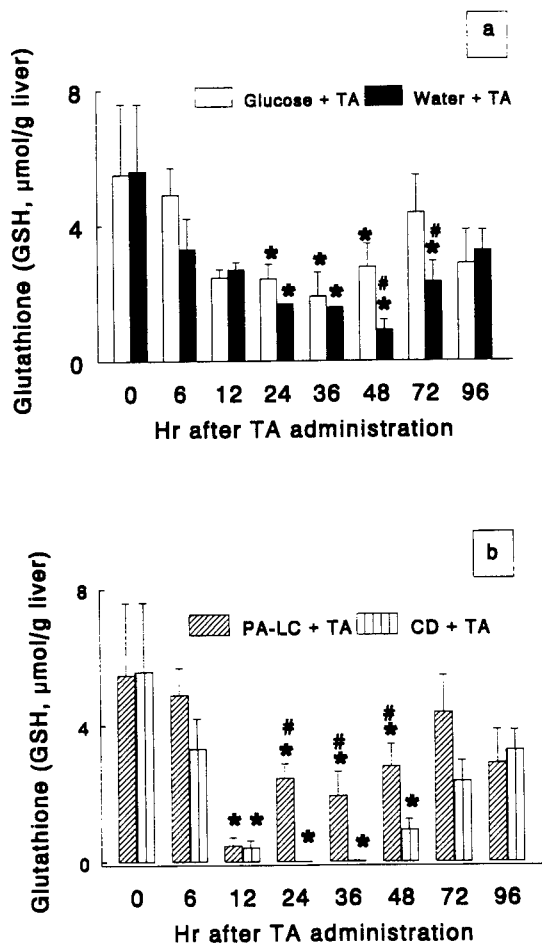


Fig. 2. (a) Hepatic reduced (GSH) levels after thioacetamide administration with or without glucose loading. Details of treatment are as in Table 1. (b) Hepatic glutathione (GSH) levels during a time-course after thioacetamide administration with or without PA-LC dietary regimen. Details of treatment are as in Table 2. Results are mean \pm S.E.M. of 4 rats for each group at each time-point. *Significantly different from control ($P \leq 0.05$). #Significantly different from the group receiving tap water and normal diet ($P \leq 0.05$).

thioacetamide (TA, Table 1, groups II and VI) experienced death (100% lethality). The other two hepatotoxicants, chloroform (50% lethality) and carbon tetrachloride (30% lethality) (Table 1, groups III & VII and IV & VIII), showed a 100% lethality in glucose-loaded rats. Since TA showed the most dramatic increase in lethality, it

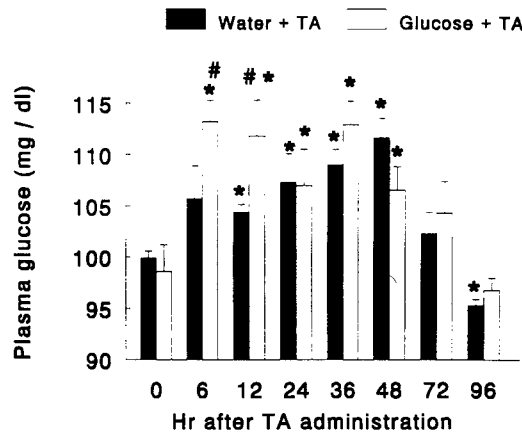


Fig. 3. Plasma glucose levels during a time-course after thioacetamide administration with or without glucose loading. Details of treatment are as in Table 1. Results are mean \pm S.E.M. of 4 rats for each group. *Significantly different from control ($P \leq 0.05$). #Significantly different from the group receiving normal drinking water and normal diet ($P \leq 0.05$).

was selected for further study. Also, lethal effects of TA were experienced by the rats between 4 to 7 days after TA administration. This model permits a better time-course study to investigate the underlying mechanism.

Plasma enzyme levels (ALT) were measured as markers of hepatotoxicity. ALT levels were the same irrespective of glucose loading (Fig. 1a) at 36 h after TA administration, which is the time of maximum injury (Chanda and Mehendale, 1995). After 36 h, progressive and nonreversible liver injury was evident through progressive elevation of ALT in rats receiving glucose, while a regression of liver injury was evident through declining elevations of ALT in rats not receiving glucose. It is noteworthy that persistent and higher elevations of ALT occurred after 36 h in glucose loaded rats, while these enzymes recovered to normal values during this time frame in rats not receiving glucose.

Hepatic glutathione (GSH) levels were decreased as a consequence of bioactivation of TA to sulfoxide or sulfone metabolites. If bioactivation of TA is decreased by glucose loading, one should expect this difference to be reflected in retained GSH in liver, particularly during the

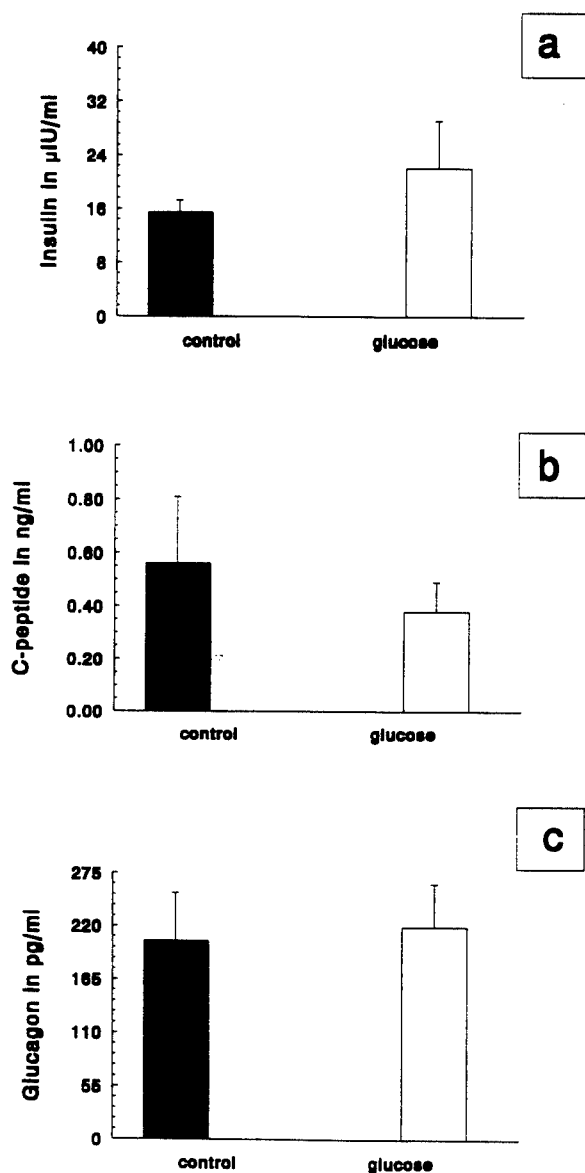


Fig. 4. Plasma insulin (a), C-peptide (b) and glucagon (c) levels in rats with or without 7 days of glucose loading. Details of treatment are in Table 1. Results are mean \pm S.E.M. of 4 rats for each group.

inflictive phase of liver injury. Therefore, hepatic GSH levels were measured to determine if any significant differences in metabolic activation of TA were evident subsequent to loading. Glucose loading did not affect the initial GSH levels (Fig.

2a). The levels of GSH decreased substantially by 24 h irrespective of the dietary regimen. The recovery from GSH depletion began at 48 h for the group on glucose loading. In the control group recovery did not start until 72 h. Thus, glucose loading neither prevented nor increased TA-induced depletion of GSH, suggesting that the mechanism by which TA inflicts liver injury is not adversely affected by this treatment.

Plasma glucose levels did not rise in glucose loaded rats (zero time point). As expected, TA treatment resulted in increased plasma glucose levels regardless of glucose loading. Since the rats were glucose loaded it was of interest to see if this could also raise the plasma glucose levels. The plasma glucose level increased significantly in rats not receiving glucose but was significantly less than the increase in rats receiving glucose until 12 h after TA administration. Therefore, the increase in plasma glucose was not significantly different between the two groups. The plasma glucose levels returned to normal by 72 h in both the groups (Fig. 3).

Absence of increased plasma glucose levels in glucose-loaded rats might be because of increased pancreatic insulin release, increased C-peptide level, or due to increased glucagon level. These three enzyme levels in plasma were measured after 7 days of glucose loading. Glucose-loading did not cause increase in these three enzyme levels (Fig. 4).

Whether the lethal effect of glucose was due to suppressed cell division and tissue repair was investigated by the proliferating cell nuclear antigen (PCNA) immunohistochemical analyses (Fig. 5). This technique helps to identify cells in different phases of the cell cycle. Normally most of the cells are in resting phase (G_0), and a relatively small number of cells are in other phases of cell cycle (data for zero time, Fig. 5), with about 3-4% cells in the G_2 phase. A decrease in G_2 cell population without any discernible increase in M phase cells was observed between 6 to 12 h in both the groups. Maximum number of cells in S phase was seen at 48 h (Fig. 5) after the administration of TA in both the groups. However, in glucose loaded rat livers, S phase stimulation was inhibited by 70%. At 72 h after TA treatment,

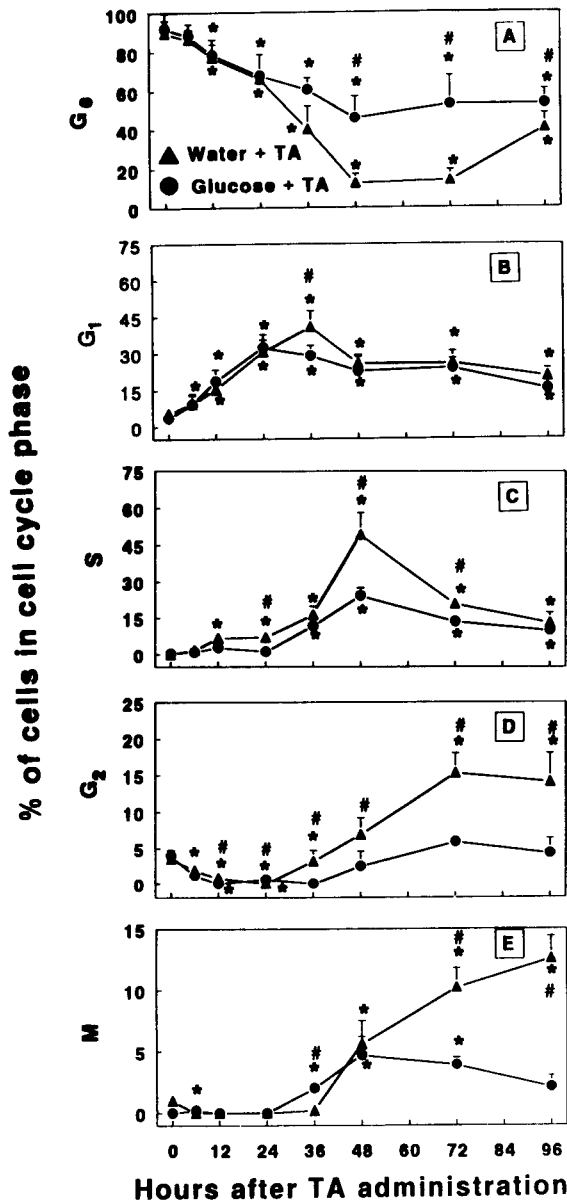


Fig. 5. Hepatocytes in different phases of cell cycle during a time-course in rats receiving thioacetamide with or without glucose loading. Details of treatment are in Table 1. Percentage was calculated from a total of 1000 viewed cells for each animal. Each time point had 4 rats per group. Thioacetamide was administered 300 mg/kg, i.p. in normal saline. The value of control did not differ from the 0 h value. *Significantly different from control ($P \leq 0.05$). #Values are significantly different from the other group at that time point ($P \leq 0.05$). Reprinted from Chanda and Mehendale (1995) by permission of FASEB J.

most of the cells had progressed to the G₂ and M phase, indicating stimulation of cell cycle progression by TA treatment. Inhibitory effects of glucose loading on TA-induced stimulation of S phase, concordant with the [³H]thymidine incorporation data (Chanda and Mehendale, 1995) and cell-cycle progression, are clearly evident from the PCNA analysis of liver sections. Consequently, glucose loading resulted in significantly attenuated mitosis (Fig. 5).

3.2. Effect of dietary palmitic acid and L-carnitine in drinking water

From preliminary studies the LD₁₀₀ dose of TA was established to be 600 mg/kg (i.p.), a dose used in these studies (Table 2). Group I, which received dietary palmitic acid and L-carnitine in drinking water, did not experience any lethality. The rats on normal diet and tap water (group II) experienced a 100% lethality in 4 to 7 days. Forty percent lethality was noted between 96 h and 120 h. All the rats died by the 7th day. Dietary palmitic acid alone (group III) or L-carnitine alone (group IV) provided only 10% protection from the lethal dose of TA. All mortalities occurred between 6 to 7 days. Approximately 30% of the rats died on day 6 and the remaining rats before the end of day 7.

Plasma enzyme level (ALT) was measured as marker of hepatotoxicity. The ALT value at 36 h for the group receiving dietary palmitic acid and L-carnitine was higher than the group receiving normal diet indicating greater liver injury at that point (Fig. 1b). After 36 h, progressive and non-reversible liver injury was evident through progressive elevation of ALT in rats receiving the normal diet, while a regression of liver injury was evident through declining elevations of ALT in rats rescued by fatty acid and L-carnitine dietary regimens. Higher liver injury in the group receiving fatty acid supplement, was of no consequence to animal survival.

Hepatic glutathione (GSH) levels were decreased as a consequence of bioactivation of TA to sulfoxide or sulfone metabolites. If bioactivation of TA is decreased by palmitic acid and L-carnitine dietary regimen, one would expect

Table 3

Total free fatty acids concentrations in the blood and in the liver of male Sprague-Dawley rats

Dietary regimen	Concentration in blood (nmol/ml)		Concentration in liver (nmol/g liver)	
	0 h	48 h	0h	48 h
Normal diet + tap water	525.9 ± 79.5	402.2 ± 43.1	21.8 ± 2.0	18.1 ± 0.9
Palmitic acid + L-carnitine	517.2 ± 50.4	352.5 ± 58.6	22.9 ± 2.7	10.6 ± 2.5*

The rats were maintained on their respective dietary regimen for the entire period of the study. Palmitic acid (8%, w/w) was admixed in the powdered rat chow and L-carnitine (2 mg/ml) was dissolved in the drinking water. On day 8 (0 h) they received a single injection of TA (600 mg/kg, i.p.). Each time point had 8 rats in each group.

*Significantly lower than the other group ($P \leq 0.05$).

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Table 4

Influence of nutritional supplementation with palmitic acid and L-carnitine on the carnitine level in blood and liver of male Sprague-Dawley rats

Dietary regimen	Concentration in blood (nmol/ml)			Concentration in liver (μ mol/g liver)		
	Short, medium and long chain acylcarnitine	Free carnitine	Total	Short, medium and long chain acylcarnitine	Free carnitine	Total
Normal diet + tap water	12.0 ± 1.1	21.1 ± 2.0	33.1 ± 2.0	0.27 ± 0.01	0.16 ± 0.02	0.43 ± 0.03
Palmitic acid + L-carnitine	11.6 ± 1.8	21.8 ± 1.1	33.2 ± 2.9	0.41 ± 0.03*	0.25 ± 0.01*	0.66 ± 0.04*

Palmitic acid (8%, w/w) was admixed in the powdered rat chow and L-carnitine (2 mg/ml) was included in the drinking water. The rats were maintained on their respective diets for 7 days. On day 8 they were killed to determine the carnitine concentration. Each dietary regimen had 4 rats.

*Significantly higher than the other group ($P \leq 0.05$).

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this difference to be reflected in retained hepatic GSH, particularly during the infictive phase of liver injury. Therefore, hepatic GSH levels were measured to determine if metabolic activation of TA was altered with palmitic acid L-carnitine dietary regimen. Palmitic acid and L-carnitine dietary regimen did not affect the initial GSH level (Fig. 2b). The levels of GSH decreased significantly by 12 h irrespective of the dietary regimens. The recovery from GSH depletion began at 24 h for the rats on PA-LC dietary regimens, whereas recovery did not start until 48 h in the control rats. Thus PA-LC dietary regimens did not prevent TA-induced depletion of

GSH, suggesting that interference with the mechanism by which TA inflicts liver injury is not the mechanism of protection.

Total free fatty acid concentration in blood and liver at 0 and 48 h are shown in Table 3. Neither the blood nor the liver concentration of free fatty acids at 0 h was significantly different between the groups. However, at 48 h the free fatty acid concentration in the livers of rats on PA-LC dietary regimen was significantly lower indicating fatty acid utilization.

Total carnitine concentration in blood and liver are shown in Table 4. There was no significant difference in carnitine concentration of

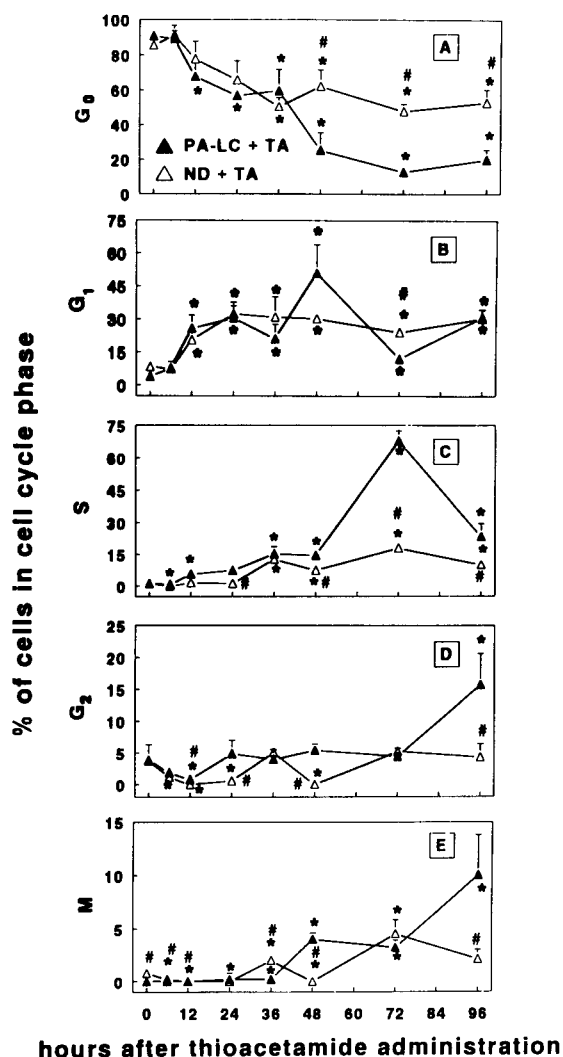


Fig. 6. Graphical representation of cell cycle progression as measured by PCNA immunohistochemical procedure. Percentage was calculated from a total of 1000 viewed cells for each animal. Each time point had 4 rats per group. Details of treatment as in Table 2. Values are mean \pm S.E.M. *Significantly different from control ($P \leq 0.05$). #Significantly different from the group receiving tap water and normal diet ($P \leq 0.05$). Reprinted from Chanda and Mehendale (1994) by permission of FASEB J.

blood in the two groups. In the liver there was a significant increase in total, free, and acyl carnitine levels in the rats on PA-LC dietary regimen.

PCNA immunohistochemical analysis (Fig. 6) was performed to examine if cell division and

tissue repair were facilitated by PA-LC dietary regimen. A decrease in G₂ cell population without any discernible increase in M phase cells was evident between 6 to 12 h in both the groups. Maximum number of cells in S phase was seen at 72 h after the administration of TA in both the groups (almost 4-fold higher in the group on PA-LC diet). At 96 h after TA treatment, most of the cells had progressed to the G₂ and M phase, indicating that cell cycle progression was stimulated by TA treatment, but the dietary supplementation with palmitic acid and L-carnitine yielded much higher mitosis (Fig. 6).

Proto-oncogene expression was examined after TA administration to rats fed either the normal diet or the PA-LC diet. Examination of proto-oncogene mRNA levels at various times after TA administration (Fig. 7) showed that *c-myc*, *v-f0*, *p53*, and *v-Ha-ras* mRNA content increased in response to TA irrespective of the dietary regimen. The messages for these proto-oncogenes were virtually undetectable in normal liver from vehicle-treated rats, but rose by 6 to 12 h and peaked by 12 to 36 h after the TA administration. No change occurred in mRNA levels for the control gene albumin among these samples. Noteworthy temporal differences were observed in the overexpression of proto-oncogenes in PA-LC rats in comparison to ND rats. In ND rats receiving TA, *p53* expression was clearly evident at 12 h and remained intense upto 48 h. In contrast PA-LC diet resulted in a preonement of *p53* expression by approximately 6 h, such that the intensity of *p53* over-expression remained between 6-36 h after the administration of TA. mRNA levels for *c-myc* were increased by 6 h and remained almost the same in both groups until 48 h when *c-myc* expression was much higher in PA-LC rats than ND rats. No significant temporal differences were evident for *v-fos* and *v-Ha-ras*.

4. Discussion

Glucose is often used as a ready source of energy for the patients with severe hepatic disorder (Price et al., 1982) without a proper under-

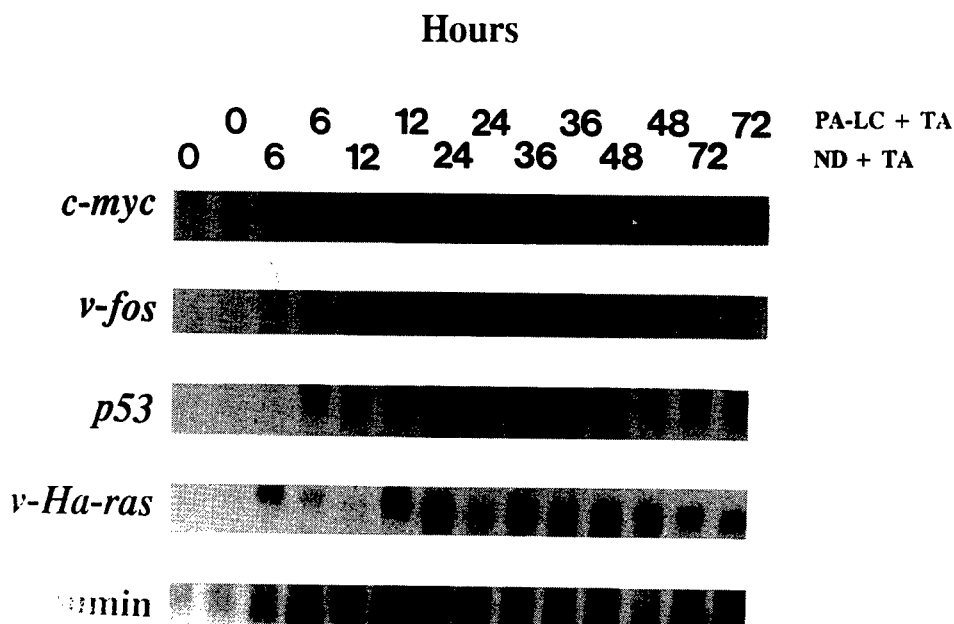


Fig. 7. Representative autoradiograms of Northern blot hybridizations of RNA isolated from rat livers on normal diet (ND) and on palmitic acid-L-carnitine (PA-LC) dietary regimen at different time points after TA administration. Details of treatment are as in Table 2.

standing of the status of glucose as an energy source after severe hepatic damage. Weinbren and Dowling (1972) and Caruana et al. (1986) demonstrated that glucose feeding may provide optimal conditions for regeneration and survival after 82-90% partial hepatectomy. However, after a 2/3 partial hepatectomy, glucose was found to inhibit [³H]thymidine incorporation suggesting inhibitory action of glucose. Additional experimental evidence is also available to suggest that glucose may be inhibitory to cell division (Bengmark et al., 1965; Ngala-Kenda and Lambotte, 1981; Holecek and Simek, 1988). It has been demonstrated experimentally that aerobic oxidation of glucose is decreased in toxic liver diseases (Simek et al., 1966; Irie et al., 1983). The metabolic block compromises hepatic glucose utilization for energetics and leads to inhibition of liver regeneration (Bengmark et al., 1965; Ngala-Kenda and Lambotte, 1981; Holecek and Simek, 1988; Chanda and Mehendale, 1995). In fulminant liver failure and related severe liver diseases, recovery may critically depend on the

availability of utilizable sources of energy in the liver. Therefore, if glucose is an inappropriate substrate to support a rapid and timely cell division and tissue repair, a reasonable and testable basis exists to hypothesize that glucose increases the toxicity of the model hepatotoxicants by inhibition of hepatocellular regeneration and tissue repair.

In this regard, there is evidence to suggest that the regenerating liver preferentially utilizes fatty acids as the source of cellular energy. Moreover, it is known that fatty acids, rather than carbohydrates, are the preferred substrates for energy-dependent metabolism in the remnant liver after partial hepatectomy (Nakatani et al., 1981). The increased demand for ATP needed to support hepatocellular division is essentially derived from fatty acid oxidation (Simek et al., 1966).

Studies were conducted to test the experimental premise that glucose is an inappropriate substrate to support cell division targeted for compensatory tissue repair needed upon centrilobular liver injury. Glucose was included in drink-

ing water for 7 days prior to a single injection of four structurally and mechanistically dissimilar centrilobular model hepatotoxicants (Table 1). Though glucose loading increased the lethality of all of the hepatotoxicants tested in this study (Table 1), maximal effect was seen with thioacetamide when a nonlethal dose became fully lethal. Hence, TA was selected for further studies with glucose and fatty acid. Based on the observations with glucose, it was hypothesized that liver injury inflicted by a lethal dose of TA kills the rats because the surviving hepatocytes lack the energy to repair the massive liver damage caused by the agent. Because TA is a centrilobular toxicant, most of the surviving hepatocytes might be expected to be in the periportal region. If recovery from massive liver injury of TA is to be expected, it can be suggested that nutritional supplementation with fatty acids, a preferred substrate for periportal hepatocytes (Jungermann and Katz, 1989), would be essential. Energy for hepatocytes to regenerate was provided by dietary supplementation with palmitic acid in the diet. A mitochondrial carrier of fatty acids (L-carnitine) was also included in the diet to facilitate the transport of the incremental palmitic acid to the mitochondria. This dietary regimen of palmitic acid (PA) with L-carnitine (LC) provided complete protection against TA lethality (Table 2, group I). Both palmitic acid and L-carnitine are essential for the protective effect since dietary supplementation of either one alone produced no significant protection. For example, palmitic acid alone in the dietary regimen resulted in only marginal reduction in TA lethality, suggesting that even though adequate levels of palmitic acid might be available through dietary supplementation, fatty acid oxidation may be limited without adequate L-carnitine to transport the incremental palmitic acid into the mitochondria. A higher hepatic content of L-carnitine and a lower fatty acid content of the liver in the protected rats support this conclusion.

One well accepted possibility for all the observed results is decreased/increased cytochrome P-450 in the liver and hence a decreased/increased formation of the toxic metabolites. Several lines of evidence are available to suggest that

increased lethality of TA upon glucose loading or decreased lethality of TA upon PA-LC dietary supplementation was not due to increased or decreased bioactivation. Glucose loading did not increase hepatic microsomal cytochrome P-450 compared to the rats not receiving glucose (Chanda and Mehendale, 1995). Hence a reasonable expectation would be a lack of increased formation of the sulfoxide and sulfone metabolite(s) of TA to increase infliction of liver toxicity. In fact, PA-LC dietary regimen caused a 2-fold increase in hepatic microsomal cytochrome P-450 compared to ND rats (Chanda and Mehendale, 1994). So, it could be expected that increased formation of the toxic metabolite(s) of TA in PA-LC rats to increase its toxicity. Accordingly, plasma enzyme data indicated greater infliction of injury at 36 h after TA administration to rats on PA-LC diet. Therefore, decreased cytochrome P-450 leading to decreased bioactivation of TA does not appear to be the mechanism of protection. The plasma enzyme (ALT) elevations (Fig. 1a) were not significantly different at 36 h in the glucose-loaded group and in the group not receiving glucose, indicating that TA-induced infliction of liver injury was not altered by glucose feeding. However, in the glucose-loaded group, the elevation of plasma enzyme significantly increased after 36 h, indicating a failure in restraining liver degeneration ultimately leading to animal death. For decreased bioactivation of TA to be the likely mechanism of protection in rats on PA-LC dietary regimen, lower elevations of these enzymes would be expected at earlier time points during the inflictive phase of injury. The ALT value for PA-LC group reached a higher point than for the control group at 36 h (Fig. 1b) indicating greater injury in PA-LC group compared to the ND group at that time point. In the ND group, the elevation of ALT progressed after 36 h indicating progression of liver injury leading to animal death. These data suggest that a difference in the bioactivation of TA is unlikely to be the critical determinant of the ultimate outcome of TA-induced infliction of liver injury. Hepatic glutathione levels have been reported to be unaffected after a low dose of TA (200 mg/kg) in male albino rats (Trennery and

Waring, 1983). In the present studies TA (300 or 600 mg/kg, i.p.) did cause a moderate depletion of glutathione (GSH), which appears to mirror the extent of TA bioactivation in view of glutathione conjugation with sulfoxide or sulfone (the active metabolites of TA) as a cytoprotective mechanism (Fig. 2a,b). As a conjecture, it might be suggested that with the dose of TA, when GSH is depleted, covalent binding of sulfoxide and sulfone metabolites of TA to macromolecules is augmented. A significantly decreased or increased TA bioactivation should be reflected in an accordingly decreased or increased GSH depletion in the rats not on glucose loading or PA-LC dietary supplementation. Our findings revealed that GSH depletion was quite similar until 36 h in both the groups irrespective of glucose loading, suggesting that decreased bioactivation of TA is unlikely to be the mechanism of protection. Likewise, GSH depletion was similar until 12 h in both groups regardless of dietary PA-LC supplementation, but was higher in the rats receiving PA-LC supplementation, once again suggesting that decreased bioactivation of TA cannot be the mechanism of protection.

The glucose levels were estimated to determine if glucose loading led to increased plasma glucose levels. Glucose loading did not affect the initial plasma glucose levels (Fig. 3). The increased blood glucose at later time-points in the glucose-loaded animals is probably because of decreased aerobic glucose utilization by the injured liver, and this corresponds to previous observations by Zivny and Simek (1989).

If palmitic acid provided as a supplement is utilized for energy production, its level should not rise in the liver and blood of protected rats. Because carnitine is used only as a carrier of fatty acid to mitochondria, carnitine levels in the liver would be expected to increase in PA-LC supplemented group. On day 8 (0 h) of the PA-LC dietary regimen, neither the total fatty acid concentration in the liver nor in blood was increased by PA supplementation (Table 3). The L-carnitine concentration was significantly higher in rats on the PA-LC diet (Table 4). At 48 h, just before the time when the maximum number of cells were in S phase, the fatty acid concentration in the

liver dropped significantly, indicating fatty acid utilization. This drop of free fatty acid concentration was significantly greater in PA-LC rats than the rats on normal diet and tap water. Hence, it can be suggested that the fatty acid supplementation along with L-carnitine as the mitochondrial carrier is necessary to meet the higher demand of energy after severe hepatic injury.

From the foregoing studies, it can be suggested that the differential outcome of liver injuries may not be attributed to differential levels of bioactivation mechanisms. These findings are more consistent with the status of cell division, and tissue repair being the likely critical mechanisms. The data suggest that stimulated hepatocellular regeneration and repair are the critical mechanisms. Administration of TA (300 mg/kg) led to the stimulation of hepatocellular regeneration at 48 h. PCNA (Fig. 6) analyses demonstrated that a significantly higher number of cells progress to the S phase at 48 h in rats not on glucose in comparison to the rats on glucose. This difference at 48 h was reflected by more numerous cells in G₂ and M phases at 72 h in the group not on glucose in contrast to the rats loaded with glucose. Therefore, it is clear that glucose loading was associated with inhibition of hepatocellular regeneration and repair of the liver tissue. Liver injury progresses in glucose-loaded rats because of insufficient cell division and tissue repair, and the rats die of liver failure. These findings also support reports by other workers that glucose inhibits hepatic DNA synthesis and hepatocellular regeneration (Holecek and Simek, 1988; Zivny and Simek, 1988). The reason for inhibition of hepatocellular regeneration remains to be investigated. One possible explanation is that glucose loading causes increased insulin release which inhibits hyperglucagonemia, which otherwise always develops after liver damage and which prevents the drop in the ATP concentration in the hepatocytes (Ngala-Kenda et al., 1984). This results in a change in the insulin/glucagon quotient to the advantage of insulin, a change known to inhibit liver regeneration. Seven days of glucose loading did not cause any significant difference in the two groups in blood insulin (Fig. 4a), C-peptide (Fig. 4b), and glucagon (Fig.

4c) levels. Also lack of hypoglycemia in these studies suggests that glucose utilization is not stymied in the glucose-loaded rats receiving TA.

An intriguing mechanism of inhibition of cell division by glucose is apparent from the elegant studies of Cerami (1985). Our observation is consistent and supportive of the previous observations by Cerami (1985) and Cerami et al. (1987) that nonenzymatic addition of glucose to nucleic acid may gradually damage the DNA. Glucose crosslinkage of DNA might be expected to decrease S phase synthesis in glucose-loaded rats.

Administration of a lethal dose of TA (600 mg/kg) led to the stimulation of hepatocellular regeneration at 72 h. PCNA (Fig. 6) analyses demonstrated that a significantly higher number of cells progress to the S phase at 72 h in PA-LC rats in comparison to ND rats. This difference at 72 h is reflected by more numerous cells in G₂ and M phases at 96 h in the PA-LC group in contrast to ND rats. Because hepatocellular necrosis and cell division are dynamic and opposing events, liver injury progresses in ND rats because of insufficient cell division and tissue repair, and the rats die of liver failure (Mangipudy et al., 1995a). The PA-LC rats survive in spite of an equivalent liver injury from TA because of the sustained and augmented cell division and liver tissue repair supported by the incremental energy made available through mitochondrial fatty acid oxidation. The importance of PA-LC dietary regimen is emphasized by the lethality study wherein neither PA alone nor LC alone provide protection against the lethality of TA. The observations support the findings by other authors that the incremental demand for ATP needed for hepatocyte proliferation after hepatic injury is provided by oxidation of fatty acids (Simek et al., 1966; Nakatani et al., 1981). It has also been pointed out by Nakatani et al. (1981) that inhibition of fatty acid oxidation significantly decreases DNA synthesis in the regenerating liver, underscoring the critical importance of the availability of cellular energy for liver regeneration.

Proto-oncogene expressions appear to play an important role in the stimulation of cellular proliferation during tissue regeneration (Bailey et al.,

1990). Liver regeneration is accompanied by a dramatic early increase in the expression of *c-myc* and other proto-oncogenes which precedes the onset of increased DNA synthesis by at least 20 h (Fausto and Shank, 1983; Goyette et al., 1983; Makino et al., 1984; Kruijer et al., 1986; Thompson et al., 1986). Blockade of *c-myc* expression by indomethacin or decadron following partial hepatectomy impedes liver regeneration suggesting a necessity of *c-myc* expression for the stimulation of DNA synthesis and cellular proliferation (Roessel et al., 1989). A higher expression of *c-myc* was seen in the rats on PA-LC diet as compared to ND rats at 48 h after TA administration though the expression was almost the same in both the groups at 24 and 36 h after TA administration (Fig. 7). Therefore, a higher *c-myc* expression at 48 h in rats on PA-LC diet might explain a higher S phase synthesis rate in these rats at 72 h compared to the rats on normal diet. Expression of *p53* started at 6 h in rats on PA-LC diet, but at 36 h both the groups had similar expression. At 48 h, the expression of *p53* was lower in rats on PA-LC diet compared to ND rats. Presumably, a higher expression of the *p53* suppressor gene helps inhibit cell division and tissue repair which took place at 72 h in the rats without PA-LC supplementation. A high expression of *v-fos* was seen in both groups at 24 h after TA administration which decreased equally in both the groups at later time points. This might indicate that expression of *v-fos* is probably not responsible for higher S phase synthesis in rats on PA-LC diet. Expression of *v-Ha-ras* was the same in both the groups from 24 h to 72 h suggesting the probability of a minimal role played by this proto-oncogene in the protection provided by PA-LC dietary regimen against TA-induced hepatotoxicity. Although definitive conclusions regarding the specific roles for overexpression of these proto-oncogene awaits additional studies, these findings suggest that cause-effect influences of proto-oncogene on hepatotoxicity are likely.

Figs. 8 and 9 illustrate the proposed conceptual framework for the mechanism of increased lethality of hepatotoxicants because of glucose loading (Fig. 8) and for the mechanism of protec-

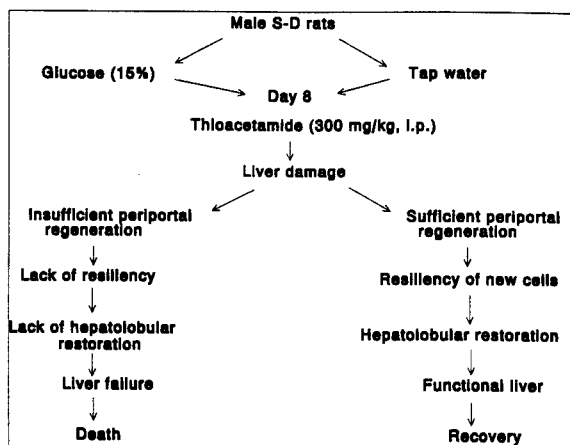


Fig. 8. A schematic representation of the proposed mechanism of increased lethal effect of the centrilobular hepatotoxicant, thioacetamide, by glucose loading.

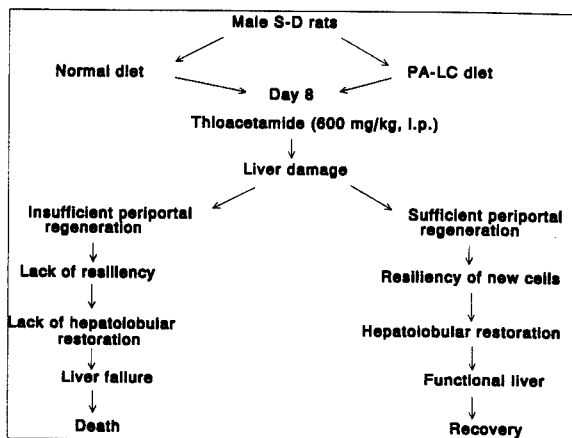


Fig. 9. A schematic representation of the proposed mechanism of protection against the lethal effect of the centrilobular hepatotoxicant, thioacetamide, by PA-LC dietary regimen.

tion by nutritional supplementation against TA lethality (Fig. 9). These findings suggest that the primary mechanism of increased lethality by glucose is a significant inhibition of cell division and tissue repair, which otherwise permits the liver tissue to overcome injury inflicted by a nonlethal dose of a hepatotoxicant (Chanda and Mehendale, 1995). On the other hand, it can be suggested that the primary mechanism of protection

against TA lethality by PA-LC dietary regimen is a significant stimulation of cell division and tissue repair, which permits the liver tissue to overcome injury inflicted by a high dose of TA (Chanda and Mehendale, 1994).

4.1. Implication to public health, biomedicine, and risk assessment

Establishing that the initial toxic or injurious events, regardless of how they are caused, can be separated from the subsequent events that determine the ultimate outcome of injury, offers promising opportunities for developing new avenues for therapeutic intervention with the aim of restoring the hormetic tissue repair mechanisms. By and large, the presently used principle is to prevent additional continuation of injury by interfering with intoxication mechanisms of toxicity through antidotal therapy. Tissue repair and healing mechanisms could be enhanced not only to block the progression of injury, but also simultaneously to augment recovery from that injury.

These findings also impact risk assessment. It is clear from these studies that nutritional status is an important determinant of the sensitivity of human populations to toxic chemicals. Although nutritional status has been recognized for quite some time as a modulator of toxicity, the decisive impact of simple dietary manipulations with glucose and fatty acid has not been known thus far. These studies illustrate that manipulations in dietary glucose can influence the outcome of hepatotoxic injury of a wide variety of toxic chemicals. Because the mechanistic basis of this modulation is inhibition of tissue repair, it can be predicted to affect a wide variety of structurally and mechanistically dissimilar toxicants.

These findings also serve to emphasize the importance of understanding the role of biological mechanisms that follow infliction of tissue injury in the outcome of that toxic injury. Inter-individual differences in the biological mechanisms may provide a scientific basis for variability in human populations. Currently in risk assessment, these biological mechanisms are not considered, even though they have decisive impact on the final outcome of toxic injury.

These findings suggest that relying only on target-organ dosimetry without including the parameters that adequately represent dynamic interplay between injury and tissue repair as opposing forces, is unlikely to yield reliable predictions of the outcome. Several studies show that progression of tissue injury is accelerated whenever cell division and tissue repair are inhibited. Such findings strongly suggest a dynamic relationship between tissue injury and tissue repair. If tissue repair is allowed to continue, this event itself would be sufficient to restrain the progression of injury and reverse the deterioration even after exposure to an ordinarily lethal dose of a chemical. Therefore, the factors that interfere with tissue repair such as nutrition (Chanda and Mehendale, 1995), other chemicals (Mehendale, 1994), and high doses of toxic chemicals (Rao et al., 1994; Mangipudy et al., 1995a) need to be identified and the biological mechanisms studied in order that this information may be included in risk-assessment paradigms. Likewise, factors that promote tissue repair, such as nutrition (Chanda and Mehendale, 1994) and early age (Dalu et al., 1995) should be studied and included in the risk assessment procedures. Inclusion of this information in the risk assessment process will likely result in further refinement of risk assessment procedures on the one hand and improvement in the estimation of risk to susceptible human populations on the other.

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SESSION IV
OCCUPATIONAL AND ENVIRONMENTAL EXPOSURE
CASE STUDIES



ELSEVIER

Toxicology 111 (1996) 181–189

TOXICOLOGY

Overview of diisocyanate occupational asthma

Jonathan A. Bernstein

*University of Cincinnati Medical Center, Department of Medicine, Division of Immunology,
231 Bethesda Avenue, M.L. #563, Cincinnati, OH 45267, USA*

Abstract

Surveillance programs established around the world have determined that diisocyanate chemicals are the most common cause of occupational asthma. In the United States approximately 100 000 workers are exposed to these chemical compounds in the workplace each year and 5–10% of these workers will develop occupational asthma. There are no known reliable risk factors or biomarkers which can be used to predict which exposed worker will develop diisocyanate-occupational asthma. Diisocyanate-occupational asthma workers manifest characteristic physiologic responses after specific bronchoprovocation which correlate with pathologic changes in their airways. However, the mechanism(s) for diisocyanate-occupational asthma remains unclear. Specific IgE antibody production to diisocyanates is found in only 10–30% of these workers. Bronchial biopsies and bronchoalveolar lavage have confirmed the presence of T-lymphocytes and eosinophils in the airways of these workers suggesting that T-cell mediated immune responses are more likely to play a central role in this disease. It is essential to diagnose diisocyanate-occupational asthma as early as possible in order to prevent or reduce the significant asthma morbidity associated with continuous long term exposure to these chemicals. Treatment of choice is removal of the worker from further exposure. Prospective studies evaluating larger populations of diisocyanate-exposed workers is essential for a better understanding of the pathogenesis and natural course of diisocyanate-occupational asthma.

Keywords: Occupational asthma; Diisocyanate; Respiratory disease

1. Introduction: low molecular weight-induced occupational asthma

1.1. Epidemiology

Occupational asthma (OA) was first recognized as early as 460 B.C. when Hippocrates described respiratory symptoms in metal workers, tailors, farmers and fishermen (Bernstein JA, 1992). In modern times, well over 200 causative agents have now been associated with asthma in the workplace (Bernstein, 1992). It is currently estimated that occupational asthma accounts for

2 to 15% of all new cases of asthma (Becklake, 1993). However, the true incidence in the general population of OA is unknown. Surveillance programs have been developed for reporting new cases. One of the better systems designed for reporting occupational respiratory diseases has been the SWORD project in the United Kingdom which stands for "Surveillance of Work-Related Occupational Respiratory Disease" (Meredith et al., 1991). The SWORD project involves reporting by pulmonologists and occupational physicians of all workers diagnosed with occupational respiratory disease. Of the 2000 cases reported after the first year, OA was

*Corresponding author.

the most common occupational respiratory disease and diisocyanates were the single most common causative agent responsible for OA (Meredith et al., 1991).

Similar programs have since been implemented in a number of countries, including the SENSOR (Sentinel Health Notification System for Occupational Risks) program in the United States, to facilitate the reporting of workers with occupational respiratory disease by primary care physicians (Becklake, 1993). These programs have been inherently limited by poor physician cooperation in recognizing and reporting new diagnoses of occupational respiratory disease. As these various systems continue to generate epidemiologic data, it is anticipated that the incidence of specific causes of occupational respiratory diseases can be more accurately assessed.

1.2. Definition of occupational asthma

Occupational asthma has been defined as "a disease characterized by airflow limitation and/or airway hyperresponsiveness due to causes and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace" (Bernstein et al., 1993). There are two categories of OA: (1) occupational asthma with a defined latency period (the time during which sensitization occurs prior to the onset of clinical symptoms); and (2) occupational asthma without a defined latency period which refers to irritant-induced asthma or the reactive airways dysfunction syndrome (RADS) (Bernstein et al., 1993).

1.3. Pathophysiology of occupational asthma

A number of low molecular weight (LMW) agents (MW < 1000 kDa) have been shown to cause OA. These LMW agents contain chemically reactive groups and act as "haptens" after binding to endogenous protein carriers (e.g. albumin, laminin or cellular membrane proteins) resulting in altered body constituents which contain one or more relevant antigenic determinants that induce an immune response. High molecular weight (HMW) antigens, such as microbial derived enzymes and animal dander, act as complete antigens and do not require conformational

change to become immunogenic (Bernstein, 1992; Bernstein, 1993b).

LMW, such as diisocyanates, induce characteristic physiologic responses after specific bronchoprovocation in patients with OA. Early asthmatic reactions (EAR), characterized by a decreased FEV₁ (forced expiratory volume in the 1st second of exhalation) 30 to 60 min after exposure to a specific agent followed by a late asthmatic response (LAR), occurring 4 to 12 h after the initial exposure are referred to as "dual" asthmatic reactions (DAR) and occur in 30 to 50% of LMW-induced OA cases. Isolated LARs occur in most of the remaining cases (Fabbri and Ciaccia, 1993). Whereas isolated early asthmatic reactions have been documented for a variety of LMW agents (ie. acid anhydrides, platinum salts and penicillium), they are very uncommon for OA induced by diisocyanate agents. These physiologic responses have been demonstrated to correlate with the pathologic changes in the airways of workers with OA. The EAR corresponds to the release of preformed and newly formed bioactive mediators from inflammatory cells in the airways (ie. histamine, leukotrienes, prostaglandins released from mast cells) (Fabbri and Ciaccia, 1993). These mediators induce bronchospasm. Many act as chemokines, which are highly specific chemoattractants for inflammatory cells that are characteristic of an asthmatic reaction (e.g. eosinophils, basophils, lymphocytes, monocytes). The LAR results from the migration of these inflammatory cells into the airways which subsequently release interleukins and cytokines important for development and perpetuation of mucous hypersecretion, smooth muscle hypertrophy and basement membrane thickening secondary to collagen deposition. Bronchial biopsy and BAL have confirmed the presence of increased numbers of eosinophils and activated T-lymphocytes during the LAR. The pathologic findings observed in OA cannot be differentiated from those changes found in non-OA (Fabbri and Ciaccia, 1993).

1.4. Mechanism of LMW protein-conjugated agent-induced OA

The mechanisms for many specific causes of LMW-induced OA have been well described,

however, underlying mechanisms for diisocyanate agents remain poorly understood. Both immunologic and non-immunologic mechanisms have been described for OA induced by LMW agents. Chemical agents, such as diisocyanates, acid anhydrides and precious metallic salts (i.e. platinum) have been documented to elicit specific IgE-mediated responses in susceptible exposed workers. As mentioned earlier, this response probably requires a "haptentation" reaction where the chemical (haptent) binds to an endogenous protein carrier in the respiratory tract (e.g. albumin or laminin) resulting in the formation of a new antigenic determinant(s) (NAD). Elevated specific IgE antibodies are found in only 10 to 30% of workers with diisocyanate OA compared to acid anhydride-induced OA where specific IgE antibodies are found much more frequently (Bernstein, 1993c). Cytotoxic mechanisms, involving complement pathway activation, have been implicated in causing hemolytic anemia and pulmonary hemorrhage in workers exposed to high concentrations of trimellitic anhydride (TMA) fumes (Zeiss et al., 1987). A late respiratory systemic syndrome (LRSS) has also been reported in TMA exposed workers and resembles an immune complex mediated reaction (Zeiss et al., 1987). Finally, cellular immune responses have also been implicated as an underlying mechanism for inducing LMW-OA. Other mechanisms proposed for LMW-induced OA include toxic damage to airway bronchial epithelial cells, neurogenic inflammation (neuropeptide-induced diisocyanate OA) and pharmacological-induced OA (organophosphates) (Bernstein, 1992).

Reactive airways dysfunction syndrome (RADS) or irritant-induced asthma is a variant form of OA (Bernstein, 1993). RADS is differentiated from the traditional form of OA in that a defined latency period of exposure prior to development of asthma is absent. Respiratory symptoms associated with RADS begin immediately after a single large exposure to an inciting agent which is usually an irritant chemical or gas such as hydrochloric acid, chlorine gas, sulfur dioxide, anhydrous ammonia and diisocyanates (Bernstein, 1993). The mechanism(s) for

RADS is unknown but appears to be mediated through non-immunologic mechanisms. Non-specific bronchial hyperresponsiveness can persist months to years after the initial onset of asthma (Bernstein, 1993).

1.5. Risk factors

One of the primary objectives of medical surveillance programs is to identify risk factors for OA induced by specific agents. Well designed medical surveillance programs should evaluate and attempt to define associations between the workers' exposure in the workplace and their clinical symptoms (Becklake, 1993). Previous studies have shown that the degree of exposure to certain causative agents is directly proportional to the worker's risk for developing OA (Becklake, 1993). Although high ambient exposure has been more commonly identified as a risk factor for OA induced by HMW allergens, exposure is also important for workers exposed to LMW agents in electronic, steel-coating and diisocyanate processes. Atopy, defined as the genetic predisposition for developing IgE-mediated hypersensitivity, has been implicated as a risk factor for many causes of HMW OA (i.e. laboratory animal workers) but not for LMW compounds, such as diisocyanates. One exception may include atopic smokers exposed to acid anhydride chemicals who appear to have a higher incidence of OA. Cigarette smoking has been identified as a risk factor for OA among platinum workers but not for diisocyanate-OA. Interestingly, a smoking history has been negatively correlated with the onset of OA induced by red cedar dust (Becklake, 1993).

2. Diisocyanates

In the United States, over 100 000 workers are exposed to diisocyanates in the workplace each year (Bernstein, 1992). The reactive N—C—O side groups of diisocyanates makes these chemicals useful in the production of polyurethane foams, elastomers, adhesives, varnishes, coatings and paint hardeners (Tse and Pesce, 1979). Diisocyanates are irritating to the mucous membranes of the eyes, nose and lungs (Kennedy and

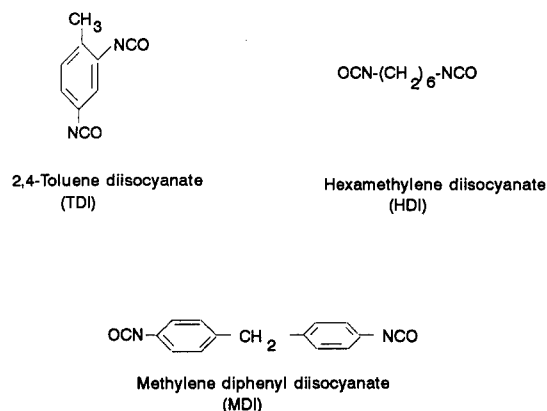


Fig. 1. Chemical structures of diisocyanates.

Brown, 1992). Respiratory disorders induced by diisocyanates have included bronchiolitis obliterans, hypersensitivity pneumonitis and occupational asthma (Kennedy and Brown, 1992). Diisocyanates are the most frequent cause of OA. It is estimated that 5 to 10% of diisocyanate-exposed workers will develop OA worldwide each year. The three most commonly used diisocyanate compounds in industry are hexamethy-

lene diisocyanate (HDI), 4-4-diphenylmethylenediisocyanate (MDI) and toluene diisocyanate (TDI) (Fig. 1) (Butcher et al., 1993). Therefore, it is not surprising that these specific diisocyanate compounds are responsible for the majority of the OA cases reported each year (Butcher et al., 1993). Each of these agents has unique chemical properties which differentiate one from the other. For example, TDI and HDI are volatile at room temperature whereas MDI requires heating above 60°C before emitting respirable vapor fumes (Kennedy and Brown, 1992). Table 1 summarizes the chemical properties of these most commonly used diisocyanate chemicals (Kennedy and Brown, 1992). Although the chemical properties of diisocyanate compounds vary significantly, workers sensitized to one compound have been demonstrated to elicit specific in vitro IgG and IgE responses and bronchial hyperresponsiveness to more than one diisocyanate chemical compound indicating significant clinical cross reactivity among these different agents (Cartier et al., 1989). Structural differences between diisocyanate compounds are believed to account in part for the heterogeneous immune

Table 1
Chemical properties of commonly used diisocyanates^a

Property	HDI	TDI	MDI
Partial listing of uses	As crosslinking agent in preparations of materials, contact lenses, and medical absorbents, and production of polyurethane spray paints	Polyurethane coatings in floor and wood finishes sealers, paints; elastomers in coated fabrics and clay pipe seals; in adhesives; cross-lining agent for nylon 6; manufacture of polyurethane foam	Bonding rubber to Rayon and Nylon; in two-component polyurethane coating systems; to produce polyurethane lacquer coatings; production of thermoplastic polyurethane resins, millable gums and spandex fibers
Commercial formulations	Monomer, trimer (biuret)	2,4-TDI; 2,6-TDI mixtures of 80:20 and 65:35	
Boiling point	127°C at 9.8 mmHg	251°C at 760 mmHg	196°C at 5 mmHg
Melting point	-67°C	19.5-21.5°C	37°C
Molecular weight	168.22	174.15	250.27
Threshold limit value	8-h Time weighted average (TWA) = 0.005 ppm (0.034 mg/m ³)	8-h TWA = 0.005 ppm (0.036 mg/m ³), short-term exposure limit (STEL) = 0.02 ppm (0.14 mg/m ³)	8-h TWA = 0.005 ppm (0.51 mg/m ³)

^aFrom Kennedy and Brown (1992).

responses observed in workers with diisocyanate OA (Cartier and Malo, 1993).

The natural course of diisocyanate-OA is variable and symptoms may persist for years after cessation of exposure. As alluded to earlier, no identifiable risk factors have yet been identified for developing diisocyanate-OA. Atopy, smoking, preexisting asthma and gender have not been identified as predisposing risk factors (Kennedy and Brown, 1992). The amount of diisocyanate exposure required to induce asthma or other upper airway symptoms is unknown and may fall well below the currently accepted threshold limit value (TLV) set by OSHA of 20 ppb (Kennedy and Brown, 1992). Physiologic, histologic and immunologic changes have been found in animals after exposure to TDI at concentrations as low as 1 ppb (Kennedy and Brown, 1992). Diisocyanate-induced airway disease in humans has commonly resulted from large exposures such as with accidental chemical spills. However, once a worker becomes diisocyanate-sensitized and symptomatic, asthma symptoms can be reproduced after reexposure to diisocyanate concentrations as low as 1 ppb (Kennedy and Brown, 1992). Recently, Bignon et al. (1994) have reported that diisocyanate workers expressing the HLA Class II alleles DQ β 1·0503 and the allelic combination DQ β 1·0201/0301 are at increased risk for developing occupational asthma (Bignon et al., 1994). Whether this finding proves to be a true risk factor for diisocyanate-OA remains to be verified.

Rats and guinea pig animal models have been used to study the uptake and distribution of diisocyanates in the respiratory tract using ^{14}C radioactive tracers (Kennedy et al., 1989). These studies found that the majority of diisocyanate uptake occurs in the upper and lower airways, sparing the alveoli (Kennedy et al., 1989). Early *in vitro* investigations of diisocyanates in animals and humans have found that diisocyanates entering into the respiratory airways bind to endogenous protein carriers. Traditionally diisocyanate-albumin conjugates have been used for determination of diisocyanate hapten specificity and have been well characterized (Tse and Pesce, 1979). However, diisocyanates are spontaneously

reactive with a variety of amino acids. Hence, the complete epitope consisting of diisocyanate and surrounding amino acids might be generated to any protein carrier. It is still unclear whether these experimental conjugates reflect the actual immunopathogenic events which occur in the respiratory tract after diisocyanate exposure. Recently, a 70000-kDa adhesion protein, known as laminin, has been identified as one endogenous protein carrier to which TDI attaches after inhalation into the airways (Kennedy and brown, 1992).

Heterogeneous isotypic antibody responses are induced by the various diisocyanate compounds. For instance, workers with HDI-induced OA are more likely to produce specific IgE and IgG antibodies antigenic determinants of the HDI-protein conjugated agent compared to MDI workers with OA, who may produce specific IgE, IgG or IgM to MDI-protein conjugates, or TDI-OA workers who may produce specific IgG antibodies but seldom elicit any IgE antibody response to TDI-protein conjugates (Butcher et al., 1993). Serologic assays (i.e. enzyme-linked immunoabsorbent assays or ELISA) using diisocyanate-conjugated proteins yield positive results more frequently than skin testing (Gallagher et al., 1981; Kennedy and Brown, 1992). However, reliable accuracy of skin testing and ELISAs depends on using conjugates which have been carefully synthesized to avoid ligand over-substitution of the protein carrier which could otherwise yield false positive results (Tse and Pesce, 1979). Elevated levels of diisocyanate specific IgG antibodies are commonly present in both diisocyanate exposed and diisocyanate-OA unless indicating that these antibodies may be useful markers of exposure to diisocyanates. Investigators have found no relationship between elevated levels of diisocyanate-specific IgE antibodies and positive diisocyanate-specific bronchoprovocation in workers with OA (Cartier et al., 1989). This observation indicates that specific IgE antibodies are not as important as once believed in the early pathogenesis of diisocyanate-OA.

It is now believed that cell-mediated immune responses play a central role in the early pa-

thogenesis of diisocyanate-OA. The earliest reports of T-lymphocyte involvement came from work performed by Avery et al. (1969) who demonstrated lymphocyte blast transformation using peripheral blood mononuclear cells (PBMCs), from workers with diisocyanate-OA, stimulated with diisocyanate-conjugated proteins. Indirect evidence for T-cell involvement was further supported by Gallagher et al. (1981) who described leukocyte inhibitory factor (LIF) release from PBMCs isolated from workers with diisocyanate-OA after stimulation with diisocyanate-conjugated proteins. LIF is a cytokine released from mononuclear cells demonstrated to inhibit polymorphonuclear cell migration (Gallagher et al., 1981). Further evidence for T-cell involvement has been supported by the presence of increased numbers of CD8+ lymphocytes in the peripheral blood of workers with diisocyanate-OA after specific TDI bronchoprovocation (Finotto et al., 1991). Recently, the University of Cincinnati Allergy laboratory has demonstrated significant PBMC thymidine uptake, using PBMCs from diisocyanate-OA workers in response to diisocyanate-conjugated protein stimulation. However, PBMC proliferation was not uniformly observed for each worker with diisocyanate induced OA. These preliminary findings indicate that diisocyanate-induced lymphocyte proliferation is not sensitive enough to identify workers with diisocyanate-OA.

Therefore, we have postulated that workers who develop diisocyanate-OA recognize and process diisocyanate-conjugated proteins differently than asymptomatic exposed workers. To investigate this hypothesis further we studied T-cell receptor (TCR) expression of V β gene repertoires in a population of diisocyanate-OA workers (Bernstein et al., 1995). Messenger RNA was extracted from PBMCs obtained from diisocyanate-OA workers, diisocyanate-exposed asymptomatic workers and nonexposed asymptomatic workers before and after in vitro coinubation with diisocyanate-conjugated proteins for 7 days (Bernstein et al., 1995). Reverse transcriptase polymerase chain reaction (PCR) followed by PCR of cDNA was performed for each subject before and after diisocyanate stimulation. Pre-

liminary results revealed that TCRs on select T-cell subpopulations have increased baseline expression of certain V β gene segments (specifically V β 1 and V β 5) compared to the baseline repertoires of diisocyanate exposed asymptomatic workers and controls (Bernstein et al., 1995). Work is currently ongoing to confirm these initial observations. If these findings are validated, TCR V β repertoire expression may serve as a useful biomarker for differentiating exposed workers who are more susceptible to developing diisocyanate-OA.

We also evaluated the same population of diisocyanate-OA workers for the presence of shared MHC Class II DR and DQ antigens (Bernstein et al., 1995). Workers with chronic beryllium disease (CBD) of the lung have been previously reported to have increased expression of DP β 1-Glu⁶⁹, now considered a genetic marker for CBD lung disease (Richeldi et al., 1993). Recently, diisocyanate workers with the allele DQ β 1·0503 and the allelic combination DQ β 1·0201/0301 have been reported to be at increased risk for developing diisocyanate-OA (Bignon et al., 1994). However, we were unable to identify shared HLA Class II antigens among the population of diisocyanate-OA workers we evaluated (Bernstein et al., 1995).

Cytokines and mediators released by inflammatory cells recruited into the airways during the LAR are also believed important in the pathogenesis of diisocyanate-OA. For example, the lipoxigenase metabolite, LTD₄, a potent neutrophil chemotactic agent, has been detected in peripheral blood shortly after TDI bronchoprovocation (Fabbri and Ciaccia, 1993). Furthermore, leukotriene LTE₄ was found in the urine during the EAR induced by TDI but disappeared during the LAR (Fabbri and Ciaccia, 1993). Analysis of bronchial biopsies from a group of workers with TDI-induced OA compared to normal controls revealed increased number of activated eosinophils in the mucosal and submucosal layers while mast cells were confined to the epithelium (Fabbri and Ciaccia, 1993)

Histamine releasing factors are cytokines referred to as chemokines which are derived from a number of inflammatory cells, including mono-

cytes and lymphocytes and have been identified in workers with diisocyanate-OA (Herd and Bernstein, 1994). Work pertaining to these specific cytokines are discussed in more detail in the following article.

3. Clinical approach to diagnosis of diisocyanate-induced OA

The diagnosis of diisocyanate-induced OA requires a careful occupational history in order to identify the relationship between respiratory symptoms and the workplace. Workers often manifest upper and lower respiratory symptoms minutes, hours or days after starting work. Symptoms typically improve away from work and recur upon re-exposure. History may be obscured in some workers who present primarily as a LAR where symptoms may not appear until the worker is at home or away from work. Also, workers with severe chronic diisocyanate-OA may not notice demonstrable improvement of their symptoms after cessation of work for weeks, months or years. The history of pre-existing asthma must be excluded before a diagnosis of OA is made, although this does not absolutely preclude making the diagnosis if there was a long asymptomatic interval between initial asthma and exposure to diisocyanates. It is important to differentiate OA from other lung diseases, such as chronic obstructive lung disease, occupational pneumoconiosis and bronchiolitis obliterans (Bernstein, 1993a,c; Bernstein, 1992).

A detailed environmental employment history should be obtained to identify whether the worker's exposure is from fumes, dust or aerosols. Workers should be questioned whether they have been involved with accidental diisocyanate spills leading to toxic exposures that may predispose or induce OA. Material safety data sheets (MSDS) should always be obtained during the initial OA evaluation (Bernstein, 1993a,c; Bernstein, 1992). Industrial hygienists experienced in air sampling measurements in the workplace are useful resources for providing the means to quantitate a worker's exposure to specific agents, such as diisocyanates (Bernstein, 1993a,c; Be-

rnstein, 1992). However, it should be emphasized that the level of exposure required to induce diisocyanate-OA may be significantly below the accepted threshold limit values enforced by OSHA. Therefore, normal levels do not necessarily indicate the absence of significant exposure.

Confirmation of diisocyanate-OA should utilize an algorithmic approach beginning with a physician-administered validated occupational questionnaire. Objective assessment for specific bronchial hyperresponsiveness in the workplace, in correlation with diisocyanate exposure, is considered a workplace challenge and should be performed if possible (Bernstein, 1993a,c; Bernstein, 1992). Methacholine or histamine challenge testing is helpful for establishing the presence or absence of nonspecific bronchial hyperresponsiveness. These tests have a very high negative predictive value for excluding the presence of bronchial hyperresponsiveness but alone are not useful in establishing a diagnosis of OA specifically induced by diisocyanates. Serial peak expiratory flow rate (PEFR) monitoring at home and at work, over 2-3 weeks, demonstrating peak expiratory flow rate variability of >15 to 20% in association with improvement away from work, is considered an abnormal response compatible with diisocyanate-OA. Shortcomings of PEFR monitoring are that patients can falsify their readings and they do not identify specific causative agents (Bernstein, 1992). When performing PEFR monitoring, patients must be instructed to measure and record their PEFR every 2-3 h at work and at home while documenting their daily work exposure to diisocyanates and requirements for asthma medication usage (Bernstein, 1992). Specific bronchoprovocation to diisocyanates is considered the gold standard for diagnosis of diisocyanate-OA (Bernstein, 1992). This challenge should be performed by personnel experienced with diisocyanate bronchoprovocation in a controlled setting with emergency treatment readily available. Typically, these specific inhalational challenge testings have been reserved to document index cases of new causative agents for OA or for medical/legal purposes such as proving a worker's eligibility for worker's compensation or disability benefits (Bernstein,

1992). Although a positive challenge is definitive proof of diisocyanate-OA, a negative challenge does not always exclude the diagnosis as sensitivity to the agent can diminish after a period of time if a worker has been removed from the workplace. Therefore, these tests should be performed in close proximity to the time a worker is removed from further diisocyanate exposure (Bernstein, 1992).

Laboratory immunologic tests are helpful in characterizing workers suspected of having diisocyanate-OA. It is important to identify whether workers are atopic to common aeroallergens by a panel of skin tests. Specific IgG and IgE diisocyanate antibodies can be readily measured using ELISAs. However, there are currently no in vitro laboratory tests that are pathognomonic for diisocyanate-OA (Bernstein, 1992).

4. Treatment

Once a diagnosis of diisocyanate-OA is confirmed, treatment should be directed toward removing the worker from further potential harmful exposure to diisocyanates. Studies which prospectively monitored workers after they have been removed from the workplace found that their asthma persisted in direct relationship to their cumulative duration of exposure and symptoms prior to diagnosis. Other determining factors for chronicity of symptoms includes the severity of airway obstruction demonstrated by spirometry and the degree of bronchial hyperresponsiveness (Chan-Yeung and Malo, 1993). Pharmacotherapy alone is not considered a suitable alternative to removing the worker from further diisocyanate exposure. Failure to remove workers from further exposure can result in further deterioration of their asthma and possible death (Chan-Yeung and Malo, 1993). In those workers who are removed from exposure and require pharmacologic treatment of acute or chronic diisocyanate-OA, the therapy is similar to treatment of non-occupational asthma which may include selective β_2 -agonists, theophylline, cromolyn or nedocromil sodium or inhaled corticosteroids in various combinations, depending

on the severity of the worker's symptoms (Bernstein, 1993a,c).

5. Prevention

Comprehensive medical surveillance programs are essential for early detection of workers with diisocyanate-OA. These programs may also help identify risk factors of workers susceptible to developing diisocyanate-OA. Risk factors can be used to prevent future cases of OA from occurring simply by removing at risk individuals from further diisocyanate exposure. However, success of such programs depends on cooperation between health care providers, company management and labor (Bernstein, 1993b). Good industrial hygiene in the workplace should always be emphasized to minimize a worker's exposure to diisocyanates. This includes careful review of safety handling procedures of toxic chemicals such as diisocyanates and avoidance of accidents such as large chemical spills (Bernstein, 1993b). Diisocyanate exposure can be minimized by improving ventilation and filtration systems in the workplace. Continuous monitoring of ambient concentrations of diisocyanates using specially designed monitors for this purpose is also important (Bernstein, 1993b). The use of respirators are not sufficient for reducing diisocyanate exposure or preventing clinical deterioration in workers diagnosed with diisocyanate-OA (Bernstein, 1993b).

Prospective studies evaluating larger populations of diisocyanate-exposed workers is essential for facilitating research geared toward a better understanding of the pathogenesis and natural course of diisocyanate-OA. Although environmental surveillance is very helpful in reducing diisocyanate exposure in the workplace, even very low levels of exposure to these chemicals can induce OA. Therefore, more accurate detection methods are required for identifying sensitive subpopulations at risk for diisocyanate-OA.

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Characterization of histamine releasing factors in diisocyanate-induced occupational asthma

Zana L. Lummus^{*a}, Rafeul Alam^b, Jonathan A. Bernstein^a, David I. Bernstein^a

^a*Department of Internal Medicine Immunology Division, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0563, USA*

^b*Department of Internal Medicine Allergy Division, University of Texas Medical Branch, Galveston, TX, USA*

Abstract

Immunologic mechanisms contributing to diisocyanate-induced occupational asthma (OA) are poorly defined. There is a relatively low incidence of diisocyanate-specific IgE antibody responses. The frequent occurrence of delayed onset asthmatic responses in workers with diisocyanate asthma suggests a role for cellular immune mechanisms. We have shown in vitro production of antigen-specific mononuclear cell-derived histamine releasing factors (HRF) by peripheral blood mononuclear cells (PBMCs) of workers with OA. Monocyte chemoattractant protein-1 (MCP-1) and RANTES (acronym for "regulated on activation normal T expressed and secreted") are chemokines found in PBMC supernatants that express HRF activity. Diisocyanate-exposed workers were tested for diisocyanate antigen-stimulated enhancement of HRF, MCP-1, and RANTES production in supernatants of PBMCs and for serum specific IgE and IgG antibody levels to diisocyanate antigens bound to human serum albumin (HSA). PBMCs of workers with diisocyanate OA showed significantly increased production of antigen-specific HRF activity and MCP-1 (> 300 ng/ml) compared to diisocyanate-exposed asymptomatic workers ($P < 0.05$). Antigen-stimulated enhancement of MCP-1 mRNA was demonstrated by reverse-transcription PCR. RANTES mRNA and chemokine secretion (< 1 ng/ml) was also demonstrated in PBMCs, but did not show antigen enhancement in OA workers. Hapten specificity for the diisocyanate chemical to which a patient had been exposed was demonstrated for HRF enhancement and for IgG antibody reactions, but not for IgE reactions. HRF production was demonstrated in PBMC subpopulations, including lymphocytes and purified T cells. OA subjects showed increased CD8⁺ cells by immunofluorescence (mean CD4⁺:CD8⁺ = 1.2 ± 0.2). The results suggest that diisocyanate antigen enhancement of HRF and MCP-1 production are stimulated by hapten-specific T cell reactions. Since a weak association has been found between IgE antibody synthesis and induction of diisocyanate OA, the role of T cell cytokines and chemokines in the pathogenesis of OA requires further investigation.

Keywords: Histamine releasing factors; Chemokine; Occupational asthma; Diisocyanate; MCP-1; RANTES

*Corresponding author.

1. Introduction

Histamine releasing factors (HRF) were first described as substances causing histamine release from basophils, that were generated by human peripheral blood mononuclear cells (PBMCs) after stimulation by Concanavalin A or streptococcal SK/SD antigen (Thuesen et al., 1979). Subsequent studies suggested a role for HRF as soluble mediators in the late allergic reaction (LAR), that occurs hours after the early phase of an IgE-mediated reaction; since the LAR is characterized by an inflammatory cellular exudate (Lichtenstein, 1988; Kaplin et al., 1991). Increased production of PBMC-derived HRF was also implicated as an *in vitro* correlate of the clinical status of allergic asthma (Alam et al., 1987; Kuna et al., 1989), allergic rhinitis (Brunet et al., 1992) and atopic dermatitis (Sampson et al., 1989).

HRF activity was found to be expressed by known cytokines (GM-CSF, IL-3, IL-8) that were less potent as basophil activators compared to PBMC-derived HRF. The molecular structure of HRF remained elusive until investigations revealed that prodigious HRF activity is associated with low molecular weight proteins of the β - or C-C chemokine subfamily, which have chemotactic and activating properties for monocytes, eosinophils, basophils, and lymphocytes. Chemokine synthesis can be induced in immunologically active cells (lymphocytes, monocytes) and non-immunologically active cells (epithelial, endothelial, smooth muscle, fibroblasts, platelets), but their mechanism of action is highly selective for subsets of inflammatory cell types that express chemokine-specific receptors (Oppenheim et al., 1991; Baggiolini et al., 1994). Basophil activating chemokines are believed to account for at least 50% of HRF activity in PBMC supernatants. These include monocyte chemoattractant proteins (MCP-1, MCP-3), RANTES, and MIP-1 α . MCP-1 has high potency for stimulation of basophil exocytosis similar to that of anti-IgE (Alam et al., 1992; Kuna et al., 1992; Bischoff et al., 1992), is a major chemoattractant for monocytes, basophils, CD4⁺ and CD8⁺ T cells, but is non-chemotactic or activating

for eosinophils, since they do not express MCP-1 receptors (Baggiolini et al., 1994, Loetscher et al., 1994). RANTES is a relatively weak exocytotic stimulus for basophils, but is a chemoattractant for basophils, eosinophils, monocytes and CD4⁺ memory T cells (Schall et al., 1990). MCP-3 expresses the combined activities of both MCP-1 (Alam et al., 1994b) and RANTES (Dahinden et al., 1994). MIP-1 α is also a weak basophil stimulator, but has potent activity for mast cell exocytosis (Alam et al., 1994a), and preferentially attracts activated CD8⁺ T cells (Taub et al., 1993).

Diisocyanate-induced occupational asthma (OA) is generally believed to be immunologically mediated, but cannot be readily explained as IgE-mediated allergic asthma since most patients do not produce diisocyanate-specific IgE antibody. An unusual feature of diisocyanate OA is the high frequency of late onset asthmatic reactions following bronchial challenge, that are similar to late phase allergic reactions, yet often occur in the absence of an early asthmatic reaction (Bernstein and Bernstein, 1993).

Basophils, eosinophils, monocytes, and T cells have all been implicated as effector cells of bronchial asthma and the LAR. HRF cytokines and chemokines have receptor-specific chemoattractant and activating activities that are independent of humoral antibody mechanisms. To evaluate cell-mediated immune mechanisms, we initiated investigations of the incidence, immunologic specificity, and cellular sources of HRF in diisocyanate OA. Our original studies demonstrated diisocyanate antigen enhancement of HRF in PBMC supernatants of workers with diisocyanate-induced asthma. The presence of antigen-specific HRF enhancement that we measured by the HRF bioassay was significantly associated with confirmed diisocyanate-induced asthma (Herd and Bernstein, 1994). In this paper, we summarize our findings in an extended group of 32 diisocyanate-exposed workers. We also studied MCP-1 and RANTES chemokine secretion as well as mRNA production by diisocyanate-HSA antigen-stimulated PBMCs in some of these subjects.

2. Materials and methods

2.1. Reagents

RPMP 1640, Hank's Balanced Salt Solution, glutamine, penicillin-streptomycin, HEPES, and phytohemagglutinin (M form) were obtained from GIBCO Laboratories (Grand Island, NY); fetal bovine serum (FBS) from HyClone Laboratories, Inc. (Logan, UT); Dextran (200–300 average kDa), bovine serum albumin (Fraction V, crystalline), mouse monoclonals: anti-CD3-FITC, clone UCHT-1, anti-human CD7, clone 3A1, anti-human CD8, clone UCHT-4, and FITC-goat anti-mouse IgG, FITC-rabbit anti-goat IgG, goat anti-human IgA, goat anti-human IgG, goat anti-human IgM, alkaline phosphatase-goat anti-human IgG, sodium pyruvate from Sigma Chemicals Co. (St. Louis, MO); goat anti-human IgE and alkaline phosphatase-rabbit anti-goat IgG from Kierkegard and Perry Laboratories (Gaithersburg, MD); human serum albumin (American Red Cross-tested) from Baxter Healthcare Corporation (Glendale, CA); Spectrapor dialysis tubing from Spectrum Medical Industries (Los Angeles, CA); Cedarlane Low-Tox-H rabbit complement and anti-human B cells (anti-mouse IAK alloantiserum), FITC-labeled, from Cedarlane Laboratories, Ltd, Accurate Chemical and Scientific Corp. (Westbury, NY); LeucoPREP cell separation tubes, tissue culture plates, plastic petri dishes from Becton Dickinson and Company (Lincoln Park, NJ); histamine RIA immunoassay kits from Amac, Inc. (Westbrook, ME); RNAzol[™] guanidinium thiocyanate reagent from Biotecx Laboratories, Inc. (Houston, TX); human rRANTES, human rMCP-1, polyclonal rabbit anti-RANTES, monoclonal mouse anti-RANTES, polyclonal rabbit anti-MCP-1 were purchased from R & D Systems (Minneapolis, MN); goat anti-rabbit (H+L), human and mouse adsorbed, from Southern Biotechnology Associates, Inc. (Birmingham, AL); the Superscript Preamplification System from Life Technologies, Inc. (Gaithersburg, MD); *Taq* polymerase from Promega Corp. (Madison, WI); primers for MCP-1 were synthesized by Operon, Inc. (Alameda, CA); primers for RANTES were synthesized by

Genosys Biotechnologies, Inc. (The Woodlands, TX).

2.2. Subjects

Thirty-seven subjects were tested for in vitro HRF production. Information on the study population is provided in Table 1. Nineteen workers presented with work-related asthmatic symptoms associated with diisocyanate exposure (group 1; 18 males, 1 female). OA was objectively confirmed in 16 group 1 workers by physiologic means. Seven workers underwent specific bronchial provocation testing (SBPT) in the laboratory using a single blind challenge method with TDI (< 20 ppb) (Moller et al., 1986). Workplace challenges were carried out by monitoring of peak expiratory flow rates (PEFR) every 2–3 h (5–6 measurements/day) under careful supervision during waking hours while there was active exposure to diisocyanates and for variable periods away from the workplace (e.g. 2 week while at work and for 2 week while away from the workplace) or by measuring cross shift changes in forced expiratory volume at one second (FEV₁) during active diisocyanate exposure. The PEFR studies were considered to be significant if there was greater than 15% variability in the amplitude percentage of the mean (highest reading – lowest reading/mean for 24 h) on 2 out of 10 days at work with no changes on days away from work (Bernstein et al., 1993) or significant decreases in FEV₁ ($\geq 20\%$ from pre-exposure baseline) over the workshift. Asymptomatic exposed workers consisted of thirteen MDI foam operation factory workers (group 2; 3 females, 10 males). Normal control subjects were non-atopics and reported no known exposure to diisocyanates (group 3; 3 males, 2 females).

Study protocols were reviewed and approved by the University of Cincinnati Human Subjects Institutional Review Board. Before participation, informed written consent was obtained from each subject.

2.3. Diisocyanate antigens

Hexamethylene diisocyanate (HDI)-, methylene diphenyl diisocyanate (MDI)-, and toluene

Table 1
Description of diisocyanate-exposed workers and control subjects

Worker	Exposure			Clinical diagnosis	Bronchial challenge	AB ^c	HRF ^d	MCP-1 ^e
	Chemical	Duration (months) ^a	Cessation (months) ^b					
Group 1: symptomatic workers								
1	MDI	336	38	+ History OA	– (lab) ^f	IgE IgG	–	NT
2	MDI,TDI	84	0	+ History OA	– (lab)	IgE IgG	–	NT
3	MDI,TDI	48	19	OA	+(lab) LAR ^g	IgG	+	+
4	MDI,TDI	33	0	OA	+(lab) LAR	–	+	NT
5	MDI	24	23	+ History OA	– (lab)	IgG	–	NT
6	TDI	Not known		OA	+(lab) LAR	IgG	+	NT
7 ^h	HDI	17	8	OA (urticaria)	+(lab)	IgE IgG	+	NT
8	MDI	25	10	OA	+(work) ⁱ	–	+	+
9	HDI,MDI	100	32	OA	+(work)	–	–	+
10 ^h	HDI	60	1	OA	+(work)	IgE IgG	+	–
11 ^h	HD1	240	19	OA	+(work)	–	–	+
12	HD1	39	0	OA	+(work)	–	+	NT
13	HDI	12	11	OA	+(work)	IgE IgG	–	+
14 ^h	HDI,MDI	60	20	OA	+(work)	IgE	+	+
15	MDI,TDI	33	3	OA	+(work)	IgG	+	NT
16	HDI	12	11	OA	+(work)	IgE IgG	NT	NT
17 ^h	HDI	36	0	+ History OA	NT	–	+	+
18	MDI	24	0.5	+ History OA	NT	–	+	NT
19	HD1	48	2	+ History OA	NT	–	+	NT
Group 2: asymptomatic exposed workers								
20–32	MDI	variable	0	No symptoms	NT	2/13 IgE 1/13 IgG	–	–
Group 3: normal controls								
33–37	None			No symptoms	NT	–	–	–

NT, not tested; '–' = negative; '+' = positive.

^aTotal time of known exposure.

^bTime elapsed since cessation of known exposure prior to clinical assessment.

^cAntibody reactions were considered positive when OD readings were greater than 3 standard deviations above the mean of negative controls in the ELISA test.

^dHRF activity in PBMC supernatant was considered positive when diisocyanate antigen-enhanced histamine release was at least 7% greater than spontaneous release in the basophil histamine release test.

^eMCP-1 was considered positive when diisocyanate antigen stimulation produced at least a two-fold increase in chemokine concentration in PBMC supernatant.

^fSingle blind controlled laboratory challenge with TDI (< 20 ppb).

^gLate airways reaction only, suggestive of isolated late phase response.

^hSkin test positive.

ⁱWorkplace challenges consisted of PEFR measurements during periods of worksite exposure compared to periods away from work or by measuring cross shift changes in FEV₁.

diisocyanate (TDI)-conjugated human serum albumin (HSA) antigens (HDI-HSA, MDI-HSA, TDI-HSA) were prepared (Tse and Pesce, 1979) and characterized (Gallagher et al., 1981) as previously reported. In brief, isocyanate reagents (Eastman Kodak Co., Rochester, NY), either 2.44 g TDI (toluene-2,4-diisocyanate), 3.50 g MDI (methylene-di-*p*-phenyl diisocyanate), or 0.21 g HDI (hexamethylene diisocyanate), were mixed with 0.9 g HSA (human serum albumin, USP, Baxter Healthcare Corp., Glendale, CA) in a final volume of 92 ml phosphate-saline buffer (0.11 M sodium phosphate, 0.15 M NaCl, pH 7.4) and stirred under a chemical fume hood for variable periods of 15 min, 30 min, 1 h, and 8 h at room temperature ($24 \pm 1^\circ\text{C}$). Reactions were stopped by addition of 92 ml 2 M ammonium carbonate and centrifugation at $3000 \times g$ for 20 min. The supernatants, consisting of soluble isocyanate-conjugated protein, were exhaustively dialyzed against four changes of 40 vols PBS (0.01 M sodium phosphate, 0.14 M NaCl, pH 7.4), and sterilized by 0.2 μm membrane filtration. Conjugates were characterized for the isocyanate/protein mol ratio by quantitative spectrophotometric analyses with appropriate standard solutions, using a modified Gutman assay (Modesto and Pesce, 1973) for determination of TDI and MDI, a gas chromatographic method for amine bound HDI (hexamethylene diamine) using 25% Apiezon L plus 10% KOH coated on Chromosorb W-H.P. (60/80 mesh) (Sandridge, 1978) after acid hydrolysis of HDI-HSA (144 h, 6 N HCl, 110°C under vacuum), and the Bio-Rad Protein Assay (Bio-Rad Chemical Division, Richmond, CA) for HSA. Conjugates containing an average of 2–13 mol isocyanate per mol of protein were selected for study.

2.4. Isolation and characterization of PBMCs and subpopulations

Venous blood (90–120 ml) was collected at a final concentration of 0.01 M EDTA. Mononuclear cells were purified by layering 20–30 ml of anticoagulated blood onto 50 ml LeukoPREP tubes (Becton-Dickinson), containing 1.077 density gradient medium, and centrifuging at

$2500 \times g$ for 30 min. The upper layer consisting of plasma was saved for antibody tests, and mononuclear cells were collected from the interface. Cells were washed twice with Hank's Balanced Salt Solution (HBSS) by low speed centrifugation ($200 \times g$) to deplete platelets.

Purified cell subpopulations were prepared as previously described (Herd and Bernstein, 1994). PBMCs, monocytes (plastic adherent cells), T cells, and total lymphocytes were analyzed by immunofluorescence reactions with fluorescein-labeled anti-CD3, anti-CD7, and anti-human immunoglobulins; and with anti-CD8 by indirect assay, using FITC-anti-mouse immunoglobulins as the second antibody. Relative cell numbers were determined as the percent of cells stained. CD4^+ cells were determined by subtraction of CD8^+ cells from CD3^+ cells.

2.5. Measurement of HRF

Methods used for generation and assay of diisocyanate antigen-induced HRF in PBMC supernatants have been described (Herd and Bernstein, 1994). Briefly, 5×10^6 cells in 1.0 ml complete medium (RPMI with 4 mM glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 5% FBS) were added to wells of a 24-well cell culture plate and incubated with 50 μg of diisocyanate-conjugated HSA antigen (HDI-HSA, MDI-HSA, TDI-HSA, or HSA carrier alone) in 0.1 ml HBSS for 72 h, 37°C , 5% CO_2 . Supernatants were removed and dialyzed (MW 3500 cutoff dialysis tubing) vs. 100 vols HBS (0.15 M NaCl, 10 mM HEPES, pH 7.4).

HRF activity was assayed by demonstrating increased basophil histamine release (HR) when normal donor basophils were stimulated with PBMC supernatants. Venous blood from a single non-atopic donor was collected with 10 mM EDTA anticoagulant, and red cells were sedimented with Dextran 200. Peripheral blood leukocytes (PBLs) were collected, centrifuged, and washed twice with HBS, 10 mM EDTA, 0.125% HSA by low speed centrifugation ($200 \times g$) to remove platelets. Then, 0.3 ml of HRF supernatant was added to 1×10^6 PBLs in 0.1 ml HBS containing 2 mM calcium, 1 mM

magnesium, and 0.125% HSA. Cells were incubated for 1 h, 37°, in a shaking water bath, centrifuged and supernatants were collected for histamine analysis. Complete histamine release was achieved by boiling cells for 10 min. Histamine was quantitated by competitive inhibition using an enzyme linked immunosorbent assay (ELISA) (histamine EIA kit, Amac, Inc.). HRF activity was determined as % HR from the formula: [(experimental HR – spontaneous HR) (100)/complete HR – spontaneous HR].

Antigen enhancement of HRF by HDI-HSA, MDI-HSA, TDI-HSA, or HSA carrier alone was determined by subtraction of spontaneous HRF activity (in medium control PBMC supernatant) from antigen-stimulated HRF activity. HRF enhancement was considered positive when the test supernatant minus spontaneous release was $\geq 7\%$, based on the observation that HRF enhancement in normal control subjects never exceeded 7% HR. Hapten-specific (i.e. diisocyanate-hapten enhancement) HRF activity was determined by subtraction of HSA-stimulated HRF activity from diisocyanate-HSA antigen-stimulated HRF activity.

2.6. Quantitation of secreted MCP-1 and RANTES

Chemokine levels were measured in PBMC supernatants, from diisocyanate-exposed subjects, before and after challenge of PBMCs with diisocyanate-HSA antigen, using an HSA conjugate prepared with the diisocyanate chemical to which the subject had been exposed. MCP-1 was quantitated by competitive inhibition ELISA (Hornbeck, 1991), using microtiter plates coated with human rMCP-1 as the standard antigen and rabbit polyclonal anti-MCP-1 at 2 $\mu\text{g}/\text{ml}$, followed by goat anti-rabbit IgG, alkaline phosphatase-conjugated. RANTES was quantitated by antibody sandwich ELISA (Hornbeck, 1991), using microtiter plates coated with mouse monoclonal anti-RANTES (2 $\mu\text{g}/\text{ml}$), human rRANTES as the standard antigen, polyclonal rabbit anti-RANTES as the sandwich antibody, and goat anti-rabbit IgG, human and mouse adsorbed, alkaline phosphatase-conjugated for indirect assay. Data were analyzed for antigen

enhancement of chemokine synthesis by subtraction of spontaneous chemokine production from antigen-stimulated chemokine production by PBMCs.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total PBMC cellular RNA was extracted and isolated by a modification of the single-step acidic guanidinium method using RNeasy[®] B (Biotech Laboratories, Inc.) and procedures recommended in the manufacturer's protocol. First strand cDNA was generated by reverse transcription using aliquots of 5 μg RNA and the Superscript Preamplification System kit (Life Technologies) according to the manufacturer's instructions. PCR amplification was performed using 5' and 3' primer sets by methods that have been described (Alam et al., 1994). Nucleotide sequences for the primers used were MCP-1 primers: 5'-GATCTCAGTGCAGAGGCTCG-3'; 5'-TGCTTGTCCAGGTGGTCCAT-3' and RANTES primers: 5'-GCTGTCATCCTCAT-TGCTAC-3'; 5'-TCTCCATCCTAGCTCATC-TC-3' PCR products were separated by 3% agarose gel electrophoresis (Nusieve 3:1) and identified by molecular size (171 bp for MCP-1; 260 bp for RANTES) using $\phi\text{X174}/\text{HaeIII}$ molecular weight markers, and cDNA standards for MCP-1 and RANTES. Semi-quantitative PCR was carried out by amplification of MCP-1 cDNA in the presence of 0.12 μCi ^{32}P -labelled dCTP/25 μl reaction mixture. X-ray film (Kodak XAR-5 film) was exposed to the gel for 3 h and developed. The MCP-1 gel bands, identified from the autoradiogram, were then cut out and counted for ^{32}P incorporation. Mitogen and antigen enhancement of MCP-1 mRNA synthesis was analyzed by quantitation of ^{32}P dCTP incorporation in MCP-1 PCR products derived from PBMCs cultured 18 h in medium containing PHA or HDI-HSA stimulator (50 $\mu\text{g}/\text{ml}/10^6$ cells). Percent increase due to stimulation was determined from the formula: [(counts/min from stimulated cells – counts/min from non-stimulated cells) (100)/counts/min from non-stimulated cells (i.e. cultured in medium only)].

2.8. Serum antibody

IgE and IgG antibody levels to HDI-HSA, MDI-HSA, TDI-HSA, and HSA were determined by isotype-specific indirect ELISA tests (Liss et al., 1988; Sarlo et al., 1990). A monitored well assay procedure was used, in which all reactions were terminated with 1 N NaOH when a standard positive control serum achieved an OD_{410nm} of 0.6. Sera producing an OD reading ≥ 0.1 and a reaction greater than three standard deviations above the mean of sera from six non-exposed control subjects were considered positive.

2.10. Data analysis

Data are expressed as mean \pm S.E.M. Group comparisons were made using the Mann Whitney test. The Wilcoxon signed-ranks test was used for paired samples within groups. Fisher's exact test was used to analyze categorical data of subject populations. A *P* value ≤ 0.05 was considered significant.

3. Results

3.1. HRF activity in symptomatic subject populations

Clinical characteristics of workers with a history compatible with occupational asthma are

shown in Table 1. The mean values for HRF in group 1 subjects were greater than for asymptomatic exposed or non-exposed control subjects after stimulation of PBMCs with phytohemagglutinin (PHA), and diisocyanate antigens (Table 2). Antigens used were human serum albumin conjugates of hexamethylene diisocyanate (HDI-HSA), methylene diphenyl diisocyanate (MDI-HSA), and 2,4-toluene diisocyanate (TDI-HSA). Twelve of 18 symptomatic workers showed HRF enhancement when PBMCs were stimulated with HSA antigen conjugate prepared using the diisocyanate chemical(s) to which the worker had been exposed (Fig. 1). HRF enhancement by antigen was not observed in 13 MDI-exposed asymptomatic control workers or in five normal control subjects. Data analysis showed a significant association (*P* < 0.05) of HRF with a clinical diagnosis of OA confirmed by a positive laboratory or workplace challenge (Table 3). HRF was also associated with a positive history of OA (*P* < 0.0001). Specific IgE antibody production was not shown to be significantly associated with OA. Specific IgG antibody production was associated with a positive history of OA (*P* < 0.0001). No association was found between HRF and antibody production of either isotype.

Table 2
HRF Activity in PBMC supernatants of diisocyanate-exposed and control subjects^a

	Group 1 (OA, n = 19)		Group 2 (asymptomatic/exposed, n = 13)			Group 3 (normal controls, n = 5)		
	Mean	S.E.M.	Mean	S.E.M.	P ^b	Mean	S.E.M.	P ^b
Spontaneous	18.0	3.74	13.1	1.30	NS	10.2	1.93	NS
PHA	30.3	4.59	25.3	3.90	NS	22.5	9.80	NS
HSA	21.1	3.68	11.9	2.26	NS	11.7	2.39	NS
HDI-HSA	23.7	4.21	NT	NT				
MDI-HSA	30.6	5.31	11.4	1.13	<0.01	11.5	2.25	<0.05
TDI-HSA	31.1	6.24	NT			NT		
DIISO-HSA	33.8	5.29	11.4	1.13	<0.01	NA		

NT, not tested; NA, not applicable; NS, not significant.

^aStatistical data of total HRF activity in PBMC supernatants after culture of cells in medium alone (spontaneous production) or with mitogen (PHA), human serum albumin (HSA) or diisocyanate-HSA-conjugated antigens (MDI-HSA, TDI-HSA, HDI-HSA, or Diiso-HSA, i.e. diisocyanate antigen(s) to which the subject had been exposed), at a dose of 50 $\mu\text{g}/\text{ml}/10^6$ cells. HRF activity is expressed as the mean = HR \pm S.E.M., by PBLs treated with HRF supernatant.

^bSignificance of difference in means, versus group 1 (Mann-Whitney test, two-tailed *P* values).

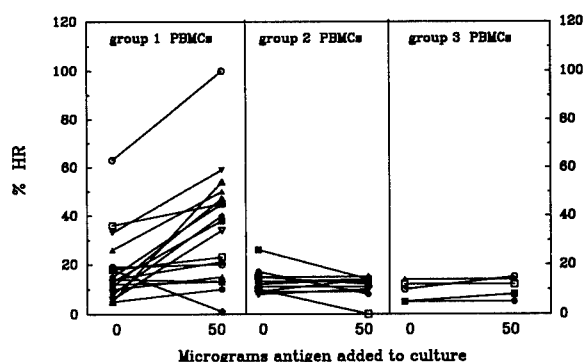


Fig. 1. Effects of antigen on the generation of HRF activity by PBMCs from 36 subjects. Results are shown for group 1 (symptomatic) and group 2 (exposed, asymptomatic) responses to 0 and 50 μ g diisocyanate-HSA antigen (HDI-HSA, MDI-HSA, or TDI-HSA) selected for the diisocyanate to which each worker was exposed in the workplace. If a worker was exposed to more than one diisocyanate, the workplace-relevant diisocyanate antigen producing the greatest HRF response is given. MDI-HSA results are shown for group 3 (non-exposed controls).

3.2. Specificity of the HRF response for diisocyanate exposure

HRF data for the 12 workers showing antigen-enhancement of HRF was analyzed to determine whether there was specificity for the diisocyanate to which the worker had been exposed.

Diisocyanate hapten-specific enhancement was determined by subtraction of HSA-stimulated HRF activity from HRF activity stimulated by diisocyanate-HSA antigens. HRF hapten-specific enhancement was determined for the diisocyanate(s) to which the worker had been exposed and compared to hapten enhancement by the diisocyanate(s) to which the worker had not been exposed. Fig. 2 shows that HRF enhancement by diisocyanate-HSA conjugates and hapten-specific enhancement of HRF were both significantly greater when PBMCs were stimulated in vitro with the diisocyanate chemicals to which the worker had been exposed.

Specificity for diisocyanate exposure chemicals was also demonstrated in the IgG (but not in the IgE) humoral immune response to diisocyanate antigens. In workers producing antibody, the mean values for IgG antibodies reacting with the diisocyanate chemical to which workers had been exposed were greater than the mean values for antibodies reacting with diisocyanates to which workers had not been exposed (Fig. 3).

3.3. Antigen enhancement of PBMC-derived HRF chemokines

MCP-1, RANTES, and HRF were measured in PBMC supernatants from eight subjects with diisocyanate-induced asthma and from seven

Table 3

Associations of specific challenge and OA history studies with in vitro specific production of IgE, IgG and antigen-enhanced HRF^a

Sample	n ^b	Categories	In vitro assays								
			HRF ^c			IgE			IgG		
			+	-	P	+	-	P	+	-	P
Subjects in group 1	15 (16)	Challenge positive	9	3	0.044	5	8	0.550	5	3	0.509
		Challenge negative	0	3		2	1		3	0	
Subjects in groups 1-3	36 (37)	Positive history OA	12	6	<0.0001	7	12	0.249	8	3	0.001
		Negative history OA	0	18		2	11		1	8	
Subjects in groups 1-3	36	HRF positive	12	0	NA	3	9	1.000	5	2	0.124
		HRF negative	0	24		5	19		4	9	

NA, not applicable; '-' = negative; '+' = positive.

^aFisher's exact test.

^bAntibody data includes one worker in group 1 that was not tested for HRF.

^cDiisocyanate-HSA enhancement of spontaneous HRF production.

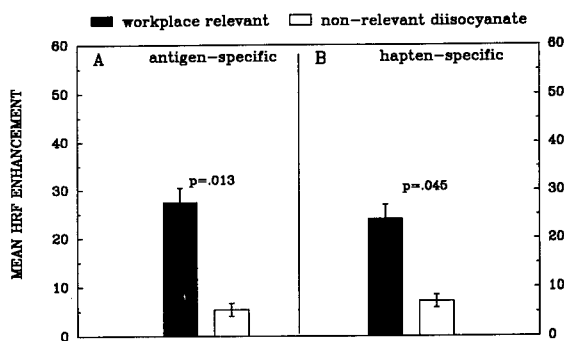


Fig. 2. Specificity of antigen-stimulated HRF production for workplace relevant diisocyanates in 12 workers showing HRF enhancement by antigen. (A) Complete antigen: the mean values were obtained after subtraction of spontaneous HRF activity from diisocyanate-HSA- (50 μ g) stimulated HRF activity. (B) Hapten component: the mean values were obtained after subtraction of HSA- (50 μ g) stimulated HRF activity from diisocyanate-HSA- (50 μ g) stimulated HRF. The amount of HRF stimulated by antigen conjugates containing the diisocyanate to which each subject was exposed (workplace-relevant) was compared to the amount of HRF produced to the diisocyanate(s) to which subjects were not exposed (non-relevant) by the Wilcoxon signs test and the level of significance is given as the two-tailed *P* value.

asymptomatic controls from groups 1 and 2, respectively. Symptomatic subjects showed higher mean production of antigen-stimulated MCP-1 (311 ng/ml) and HRF (>30% HR) than asymptomatic controls (Fig. 4). RANTES production was similar in OA subjects (1 ng/ml) and asymptomatic controls.

PBMCs of subject no. 13 with HDI-induced OA and a control subject (no. 35) were tested for mRNA for MCP-1 and RANTES by RT-PCR analysis (Fig. 5). PCR products, detected in ethidium bromide-stained gels, showed that mRNA for RANTES was present in PBMCs obtained at the time of cell isolation (T0) but was substantially decreased after 4 h of culture (T4). MCP-1 mRNA in PBMCs tested prior to culture was not detected in stained gels (T0), but was readily detected after 4 h culture. Enhancement of chemokine mRNA synthesis by mitogen or antigen, compared to spontaneous synthesis (Sp) by cells in medium alone, was not apparent for either subject by analysis of ethidium bromide-stained gels. However, quantitation of MCP-1

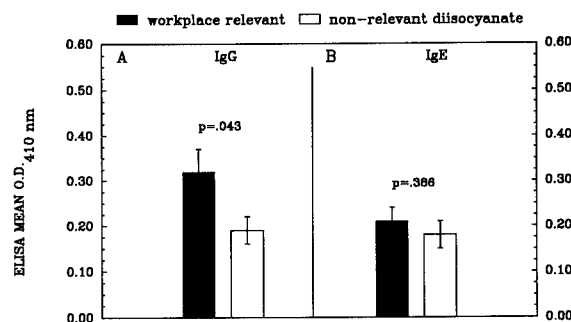


Fig. 3. Specificity of antibodies for workplace-relevant diisocyanates in, (A) 11 group 1 workers showing IgG antibody, and (B) seven group 1 workers producing IgE antibodies. The mean antibody levels were determined from optical density readings of ELISA test results. Antibody levels to the diisocyanate chemical to which each worker had been exposed (workplace-relevant diisocyanate) were compared to antibody levels to diisocyanate chemicals to which the worker had not been exposed (workplace non-relevant diisocyanate) by the Wilcoxon signs test and the level of significance is given as the two-tailed *P* value.

³²P-labeled PCR products from 18-h cultures showed greater synthesis of antigen stimulated MCP-1 mRNA in the asthmatic subject, compared to the normal control subject (Table 4). HDI-HSA stimulation of PBMCs produced a 38% increase in MCP-1 mRNA from the asthmatic subject, compared to an 18% increase in mRNA from the normal subject.

3.4. Cellular origins of diisocyanate-induced HRF synthesis in PBMCs

HRF production by purified PBMC subpopulations was determined in four subjects, consisting of three workers (nos. 8, 10, 12) with MDI- or HDI-induced OA and one asymptomatic MDI-exposed subject (no. 20). Characteristics of total PBMCs and the purified cell populations, determined by flow cytometry and reactions with fluorescent antibody reagents are shown in Table 5. B cells were demonstrated in purified lymphocytes as cells bearing surface immunoglobulin markers (sIg⁺). Purified T cells from the asymptomatic subject showed a normal CD4⁺:CD8⁺ ratio (2.2), while low CD4⁺:CD8⁺ ratios (1.0–1.4) were found in each of the OA patients, suggesting that CD8⁺ (suppressor/cytotoxic) cells were increased.

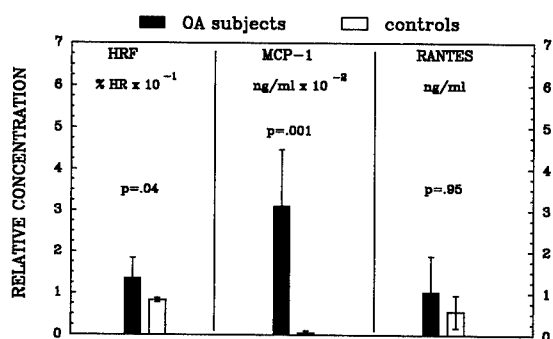


Fig. 4. Enhancement of HRF and chemokine production from PBMCs by diisocyanate-HSA conjugated antigens derived by subtraction of spontaneous production from diisocyanate-HSA-stimulated production. (A) HRF activity, measured as = HR in the basophil histamine release assay, and reduced by a factor of 10. (B) MCP-1 measured in ng/ml and reduced by a factor of 100. (C) RANTES measured in ng/ml. The mean values for eight patients with diisocyanate-induced asthma are compared to seven asymptomatic controls by the Mann-Whitney test and the level of significance is given as the two-tailed *P* values.

The mean HRF response of the PBMC subpopulations is shown in Fig. 6. The results showed increased production of HRF by monocytes, lymphocytes, and T-cells. Purified T cells (B cell depleted) incubated in medium alone showed high spontaneous release of HRF. Reduced amounts of HRF were detected in diisocyanate antigen-stimulated T cell cultures, suggesting that T cell spontaneous release of HRF was downregulated by antigenic stimulation. Spontaneous release of T cell HRF was also reduced by PHA stimulation (data not shown). Spontaneous release of HRF did not occur in cultures of the whole lymphocyte preparations from which the T cells had been purified. It appears likely that non-T cells or cytokines generated in the lymphocyte cultures produced inhibition of spontaneous production of T cell HRF. Antigen specific enhancement of HRF was clearly demonstrated in whole lymphocyte cultures. Antigen enhancement of HRF was observed in only one monocyte culture, from subject no. 12 (results not shown). This monocyte preparation may have contained adherent B cells, since 10% of the cells showed immunofluorescent staining

RT-PCR ASSAY FOR mRNA SIMULTANEOUS ANALYSIS FOR MCP-1 & RANTES

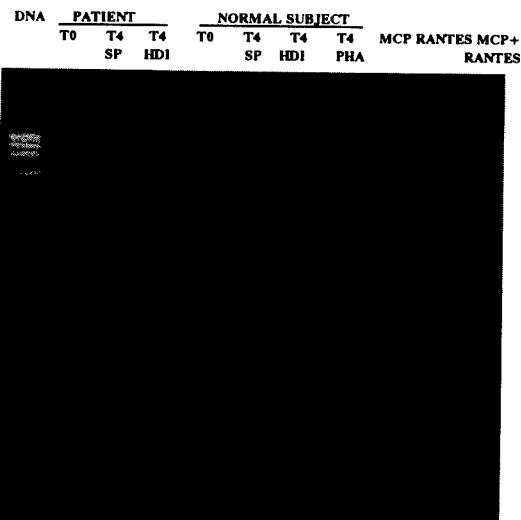


Fig. 5. Simultaneous analysis for MCP-1 and Rantes mRNA expression in PBMCs from a patient with diisocyanate-induced OA and a normal subject. PBMCs were tested prior to culture (T0) and after 4 h of culture (T4) in medium alone (RPMI, 5% FBS), or in medium containing mitogen (PHA) or antigen (HDI-HSA). mRNA was reverse-transcribed, and cDNA was amplified by PCR, using primers for MCP-1 and RANTES. The ethidium bromide-stained gel of PCR products shows that RANTES and MCP-1 mRNA were present in freshly isolated PBMCs and that cultured cells showed increased MCP-1 mRNA and decreased RANTES mRNA. Positive controls for the amplified products of MCP-1 (171 base pairs) and RANTES (260 base pairs) are shown.

for surface immunoglobulin (Table 5). Alternatively, the sIg⁺ reaction could have been due to cytophilic antibodies bound to monocyte Fc receptors, since subject no. 12 was an antibody-producing subject. T cell recognition of diisocyanate antigen was strongly suggested by the decreased production of spontaneous HRF in T cell cultures exposed to antigen, as well as by antigen enhancement of lymphocyte HRF in non-antibody-producing subjects (nos. 8 and 10).

4. Discussion

These studies have demonstrated that peripheral blood mononuclear cells (PBMCs) of

Table 4
PCR analysis of antigen and mitogen enhancement of MCP-1 mRNA synthesis in PBMCs of subject with HDI-induced OA

Subject/group	PBMC stimulator	Counts/min in PCR ³² P-MCP-1	% Increase in MCP-1
No. 35/group 3	Medium control	7027	0
	PHA	9424	34.1
	HDI-HSA	8347	18.8
No. 13/group 1	Medium control	9203	0
	PHA	13 536	47.1
	HDI-HSA	12722	38.2

Peripheral blood mononuclear cells (PBMCs) (10^6 /ml) were cultured in medium alone (RPMI, 5% FBS), or in medium containing mitogen (PHA) or antigen (HDI-HSA) for 18 h. MCP-1 mRNA was reverse-transcribed, and cDNA was amplified by the polymerase chain reaction (PCR) in the presence of ³²P-dCTP. ³²P-MCP-1-amplified DNA formed a single band in agarose gel electrophoresis. MCP-1 gel bands were cut out and ³²P incorporation was measured as counts/min minus background. Percent increase in MCP-1 due to mitogen or antigen enhancement was determined from MCP-1 DNA using the formula: [(counts/min from stimulated PBMCs - counts/min from medium control PBMCs) × 100]/counts/min from medium control PBMCs.

workers with confirmed diisocyanate-induced occupational asthma can be stimulated in vitro with diisocyanate-HSA antigens to produce basophil-activating histamine releasing factors (HRF) and monocyte chemoattractant protein 1 (MCP-1). Our previous studies of 19 subjects,

that had been exposed to hexamethylene diisocyanate (HDI), methylene diphenyl diisocyanate (MDI), or toluene diisocyanate (TDI), showed significant antigen enhancement of HRF production in workers with clinical histories consistent with diisocyanate-induced occu-

Table 5
Characteristics of PBMCs and purified cell subpopulations

Subject no.	Percentage of cells tested			
	20	8	10	12
PBMC profile^a				
Lymphocytes	78.6	74.7	85.2	77.5
CD3 ⁺	70.0	80.0	79.0	77
CD7 ⁺	81.0	72.0	78.0	73
Monocytes	16.2	19.9	13.5	18.8
Granulocytes	5.2	5.4	1.3	3.7
Purified subpopulations^b				
Lymphocytes sIg ⁺	<1	<1	7.0	5.0
T cells CD3 ⁺	>99	>99	>99	>99
sIg ⁺	<1	<1	<1	<1
CD4 ⁺ :CD8 ⁺ ^c	2.2	1.4	1.2	1.0
Monocytes sIg ⁺	<1	<1	<1	10.0

^aPeripheral blood mononuclear cells (PBMCs) were purified by density gradient sedimentation. The cellular profile of PBMC preparations (lymphocytes, monocytes, granulocytes) was determined by the use of a flow cytometer.

^bMonocytes were purified by adherence to plastic tissue culture dishes. Non-adherent cells (lymphocytes) were treated with antisera to immunoglobulins plus complement to obtain B cell-depleted lymphocytes (T cells). Specific immunofluorescence reactions were used to detect T cell surface antigens (CD3, CD7, CD8) and B cell surface immunoglobulin (sIg⁺).

^cThe CD4⁺:CD8⁺ ratio was determined as: CD3⁺ cells minus CD8⁺ (equal to CD4⁺ cells) divided by CD8⁺ cells.

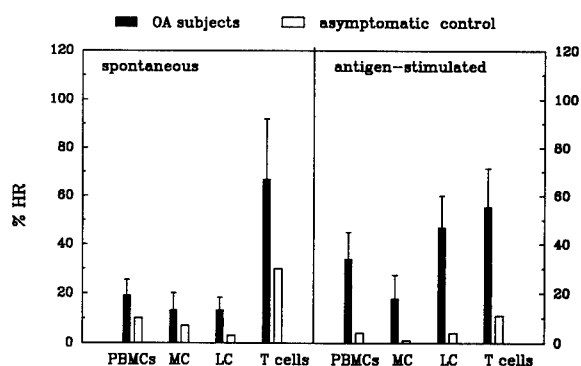


Fig. 6. HRF production (% HR) by un-fractionated PBMCs and monocytes (MC), lymphocytes (LC), and T cells purified from PBMCs of four diisocyanate exposed workers. Cell supernatants were tested for HRF after 18-h incubation either in medium alone (spontaneous) or in medium containing diisocyanate-HSA antigen (antigen-stimulated). Mean HRF production by cells from three subjects with diisocyanate-induced OA is compared to HRF production by cells from an asymptomatic control.

pational asthma (OA), compared to exposed asymptomatic workers or non-exposed controls. In workers with a positive history of OA, antigen-specific HRF enhancement was shown to be associated with a positive response to specific bronchial provocation testing or to workplace challenge (Herd and Bernstein, 1994). In this paper, our previous findings have been confirmed in a larger group of 32 workers.

Immunological factors that contribute to diisocyanate-induced asthma have not been defined. The disease develops in about 5% of exposed workers, and atopy has not been shown to be a risk factor (Bernstein and Bernstein, 1993). Most cases of diisocyanate-induced OA are not mediated by IgE antibodies (Cartier et al., 1989). Despite the finding that 37% of workers in our symptomatic study population produced IgE antibodies and 58% produced IgG antibodies, there was no association between antibody production and confirmed OA.

Previous studies in sensitized workers have demonstrated humoral cross-reactions between different diisocyanate antigens (Baur, 1983). Interestingly, we found that HRF responses in individual workers were elicited by antigen con-

jugates containing occupationally relevant diisocyanates (i.e. determined to be present at the subject's worksite) as well as non-relevant diisocyanates. However data analysis showed that the magnitude of the mean HRF response to occupationally relevant diisocyanates was significantly greater than the HRF response to non-relevant diisocyanates, to which workers were not exposed. The hapten-specific component of the HRF response also showed greater specificity for occupationally relevant diisocyanates. Four of the symptomatic workers (nos. 3, 7, 8, 14) in this study showed antigen specific HRF responses after they had been removed from work exposure for 8-20 months. Thus, the HRF response in diisocyanate-induced asthma shows memory and specificity indicative of a cell-mediated immune mechanism.

Our studies of the cellular sources of HRF showed that lymphocytes were the most active producers of antigen-stimulated HRF. Purified T cells showed high spontaneous release, suggesting *in vivo* activation. No spontaneous release occurred in cultures of the whole lymphocyte preparations from which the T cells were purified. It is therefore possible that T cell production of HRF was inhibited or down-regulated by other cells in the whole lymphocyte preparations. Stimulation of purified T cells with antigen also had the effect of down-regulating HRF production. High spontaneous production of HRF by T cells has not previously been reported. In studies of purified cell populations from normal subjects or ragweed-sensitive asthmatics, monocytes and B cells were found to produce both spontaneous HRF and allergen-induced HRF, while T cells produced HRF only after stimulation with mitogen or SK/SD, a delayed hypersensitivity recall antigen (Goetzl et al., 1984; Alam et al., 1989; Turner et al., 1991). Of the chemically characterized cytokines with HRF activity that are known to be produced by lymphocytes, T and B lymphocytes have been reported to produce GM-CSF (Zupo et al., 1992) and MIP-1 α , while T lymphocytes also produce RANTES and IL 8 (Baggiolini et al., 1994). We did not find that purified monocytes were a major source of antigen-stimulated HRF.

Quantitation of MCP-1 and RANTES showed diisocyanate antigen stimulation of MCP-1 secretion by PBMCs of subjects with diisocyanate-induced asthma, compared to asymptomatic control subjects. RANTES was present at 100-fold lower concentrations than MCP-1 in supernatants of PBMC cultures, and was not increased in PBMCs of symptomatic subjects, compared to asymptomatic subjects. Our limited investigations of chemokine mRNA synthesis in two subjects showed that RANTES mRNA was present at relatively high levels in freshly isolated PBMCs, compared to cultured PBMCs, and did not appear to be increased by specific antigen stimulation. MCP-1 mRNA appeared to be increased in cultured cells, compared to freshly isolated cells, and to show diisocyanate-specific stimulation in PBMCs of a symptomatic subject. The lower amount of HDI-HSA stimulation of MCP-1 mRNA that was found in normal human PBMCs is possibly due to the HSA carrier protein. Some normal and symptomatic subjects show a low level of HSA enhancement of HRF. MCP-1 is a major product of monocytes, and is not produced by lymphocytes (Baggiolini et al., 1994). Therefore, antigen-specific stimulation of MCP-1 in PBMC cultures would require either activation of monocytes by lymphokines, or cytophilic antibodies bound to Fc receptors.

Our observations suggested increased production of CD8⁺ (suppressor/cytotoxic) T cells in the peripheral blood of diisocyanate OA subjects, as has previously been reported by others (Finotto et al., 1991). This finding is consistent with the chemokine profile that we demonstrated in PBMC supernatants. MCP-1 has chemoattractant activity for both CD4⁺ and CD8⁺ cells (Loetscher et al., 1994), while RANTES has been shown to be selectively chemotactic for CD4⁺ memory T cells (Schall et al., 1990). Our studies do not rule out the possibility that other cytokines and chemokines, e.g. MIP-1 α , which selectively recruits activated CD8⁺ cells (Taub et al., 1993), may also contribute to HRF activity.

Increased numbers of CD8⁺ T cells in diisocyanate-induced asthma may be relevant to the low incidence of IgE antibody synthesis in symptomatic workers. Cloned T cells derived

from bronchial mucosa of diisocyanate asthmatics have been predominantly CD8⁺ cells (82%) all showing production of IFN- γ (Maestrelli et al., 1994), which is known to suppress IgE antibody production in vivo (Finkelman et al., 1988) and to also increase MCP-1 gene expression (Gruss et al., 1994). Studies have suggested that the inhalation pathway of antigen exposure preferentially induces CD8⁺ cells and CD4⁺ Th-1 cells and results in selective suppression of IgE antibody (McMenamin and Holt, 1993).

The possibility that the induction of diisocyanate asthma is associated with MHC class I restricted T cell reactions requires further investigation. In animal models, hapten-specific T cells have been difficult to demonstrate, and it is generally assumed that T cells recognize epitopes of haptened proteins in the context of a peptide/MHC ligand (Nalefski and Rao, 1993). In addition, CD8⁺ T cells generally have been shown to recognize endogenously synthesized antigens, while CD4⁺ T cells recognize exogenous antigens (Brodsky and Guagliardi, 1991). However, CD8⁺ cells with specificity for exogenous antigens have been demonstrated (Rock et al., 1993; Hisatsune et al., 1995). Whether hapten presentation by antigen presenting cells requires intracellular processing is currently unresolved, since peptide digests of haptened proteins have shown T cell stimulation after binding to MHC class I molecules of glutaraldehyde-fixed cells (Ortmann et al., 1992).

HRF and related chemokines could contribute to the immunopathogenesis of diisocyanate-induced OA through their chemoattractant specificity for subsets of inflammatory cell types. Bronchial asthma is characterized by cellular infiltration of monocytes, T lymphocytes, and eosinophils into the bronchial mucosa (Azzawi et al., 1990; Poston et al., 1992). MCP-1 specifically attracts and activates monocytes, and has been shown to be present in the subepithelium of asthmatic airways at increased levels, compared to normal subjects (Sousa et al., 1994). RANTES is selective for eosinophils (Ebisawa et al., 1994) and has been found in the BAL fluid of asthmatics at higher than normal levels (Alam et al., 1994c). A comparative study of the bronchial

mucosa in diisocyanate-induced asthma and in atopic (extrinsic) asthma did not show any distinctive differences in the patterns of inflammatory cell infiltrates (Bentley et al., 1992). In bronchial biopsies, the basement membrane of asthmatics, compared to normals, showed significant increases in cell numbers of eosinophils and IL-2R⁺ T cells, but not in numbers of CD3⁺, CD4⁺, or CD8⁺ T cells, neutrophils, or macrophages. Other studies have shown increased numbers of total mononuclear cells, degranulated eosinophils and mast cells in the lamina propria, beneath the basement membrane, in subjects with diisocyanate OA, compared to normals (Saetta et al., 1992). It seems clear that eosinophils are major effector cells of diisocyanate-induced asthma. Our failure to induce antigen-stimulated RANTES secretion in cultured PBMCs does not preclude that this chemokine is produced locally in the airways or that other cytokines and mediators may be important in stimulation of eosinophilic migration and activation as a response to injury.

In conclusion, the HRF bioassay appears to detect a cell-mediated immune response to the diisocyanate hapten in subjects with diisocyanate-induced asthma. At least some of the long-lasting effects of diisocyanate-induced OA are likely to be explained as specific T cell sensitization to the diisocyanate hapten after chemical binding to an endogenous carrier protein.

Acknowledgements

Susan Stafford (UTMB at Galveston) and Shervin Yermian (UC, Cincinnati) provided excellent technical assistance. This work was supported by NIOSH Grant No. K01 OH00073 and NIEHS Grant No. ES06096-02A.

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Toxicology 111 (1996) 207-212

TOXICOLOGY

Development and application of non-invasive biomarkers for carcinogen-DNA adduct analysis in occupationally exposed populations

G. Talaska^{*a}, J. Cudnik^a, M. Jaeger^a, N. Rothman^b, R. Hayes^b, V.J. Bhatnagar^c,
S.J. Kayshup^c

^a*The Department of Environmental Health, University of Cincinnati, Cincinnati, OH 45267-0056, USA*

^b*Environmental Epidemiology Branch, Occupational Studies Section, The National Cancer Institute, Bethesda, MD, USA*

^c*National Institute for Occupational Health, Amembabad, India*

Abstract

Biological monitoring of exposures to carcinogenic compounds in the workplace can be a valuable adjunct to environmental sampling and occupational medicine. Carcinogen-DNA adduct analysis has promise as a biomarker of effective dose if target organ samples can be obtained non-invasively. We have developed non-invasive techniques using exfoliated urothelial and bronchial cells collected in urine and sputum, respectively. First morning urine samples were collected from 33 workers exposed to benzidine or benzidine-based dyes and controls matched for age, education, and smoking status. Sufficient DNA for ³²P-postlabelling analysis was obtained from every sample. Mean levels of a specific DNA adduct (which co-chromatographed with standard characterized by MS) were elevated significantly in the benzidine-exposed workers relative to controls. In addition, workers exposed to benzidine had higher adduct levels than those exposed to benzidine-based dyes. This study demonstrates the usefulness of these non-invasive techniques for exposure/effect assessment. To be useful in occupational studies, biomarkers must also be sensitive to exposure interventions. We have conducted topical application studies of used gasoline engine oils in mice and found that the levels of carcinogen-DNA adducts in skin and lung can be significantly lowered if skin cleaning is conducted in a timely manner. The combination of useful, non-invasive techniques to monitor exposure and effect and industrial hygiene interventions can be used to detect and prevent exposures to a wide range of carcinogens including those found in used gasoline engine oils and jet exhausts.

Keywords: Non-invasive biomarkers; Carcinogen-DNA adduct analysis; Occupational hazards; Exposure/effect assessment; Monitoring technique

*Corresponding author.

1. Introduction

Controlling exposure to chemical carcinogens in the workplace is difficult from the point of view of the industrial hygienist because although airborne exposure limits exist, only very low levels are allowed. In addition, many carcinogenic compounds are well absorbed through the skin. Therefore, dermal exposure may account for a significant fraction of total dose, yet not be taken into account when only air sampling is done. Biological monitoring is a reasonable alternative to air sampling under these circumstances. Biological monitoring for carcinogens exists on several levels. These are illustrated in Fig. 1 as part of a proposed linear continuum between external exposure and disease. If the concern centers around the question of whether exposure has occurred, then a marker of internal dose is sufficient and usually the easiest biological marker to obtain. If there is an indication that there are individual differences in response to an internal dose then a marker of effective dose such as carcinogen-DNA adducts, or early effects, such as mutation frequency, may be more appropriate. In the same regard it is more useful to obtain data on the level of the marker in the target tissue rather than a surrogate tissues such as blood

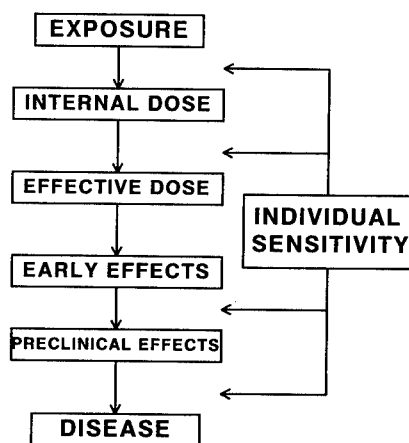


Fig. 1. Continuum between exposure and disease with potential biomarkers of naturalistic events indicated.

lymphocytes because there might be a significant difference in response to the carcinogen.

Measurement of carcinogen-DNA adducts integrates differences in absorption, metabolism and, if the levels in the target organ can be measured, distribution of the reactive form of the compound. While carcinogen-DNA adducts are the ultimate mutagens, there is a low probability that a particular carcinogen-DNA adduct will produce a neoplasm, due to the size of the genome and the relative small size of the critical targets. Carcinogen-DNA adduct measurements may, therefore, have a role in disease prevention. If adducts are detected and subsequent exposure terminated, then presumably there will be a reduced probability of disease. If, however, there is chronic, uninterrupted exposure to the carcinogen prior to the adduct measurement, then the reduction in potential risk would be small as disease is probably a function of total area of cumulative lifelong DNA damage.

Sensitive techniques have been developed to monitor carcinogen-DNA adducts in human tissues. Among the most useful is ^{32}P -postlabelling (see Talaska et al., 1992a for review). This technique has proven useful in experimental models with animals exposed to a wide variety of carcinogens. It is an extremely sensitive technique; adducts have been detected reproducibly at levels of from 1-10 per genome, although the absolute recovery of each adduct likely differs. And, when used in conjunction with other analytical techniques, ^{32}P -postlabelling has been used to chemically identify certain DNA adducts in human tissues (Talaska et al., 1991a; Zeisig and Möller, 1995). In this paper we discuss the use of ^{32}P -postlabelling to monitor exposure to carcinogens in the human urinary bladder.

Most carcinogens encountered in occupations affect organs which are usually thought to be unamenable to non-invasive sampling. A list of some of these agents and their target organs are found in Table 1. Lung and urinary bladder are two major targets for occupational carcinogens. And, while it is difficult if not impossible to obtain samples of tissues from these organs non-invasively, we reasoned that since cells from the lung and urinary bladder regularly exfoliate, it

Table 1
Human target organs for several occupational and environmental carcinogens

Carcinogen	Target organ
Aflatoxin	Liver
4-Aminobiphenyl	Urinary bladder
Benzene	Hematopoietic
Benzidine	Urinary bladder
Bis(chloromethyl)ether	Lung
Isopropyl oils	Nasal cavity, larynx
2-Naphthylamine	Urinary bladder
Benzo[<i>a</i>]pyrene	Lung, skin
Vinyl chloride	Liver, lung

might be possible to collect these cells and use them to monitor exposure and effects within the tissue. Although estimates vary considerably, the lifespan of both urinary bladder and lung cells has been reported as being about 100 days (Clayson and Lawson, 1987; Cotes and Steel, 1987).

2. Experimental validation

The exfoliated urothelial cell technique for DNA adduct analysis was developed in a dog model. This species was chosen because dogs are non-acetylators and therefore model the effects in the group of humans, slow acetylators, thought to be at greatest risk for aromatic amine-induced urinary bladder cancer. The animals were treated with 5 mg/kg 4-aminobiphenyl orally, 5 days per week and for as long as 10 weeks. The complete methods and results of this study have been presented earlier (Talaska et al., 1990). The salient findings were that specific 4-aminobiphenyl adducts were detected in the exfoliated urothelial cells of the animals very soon after treatment. These results suggest that exposure to these cells was intraluminal, a finding that corroborates other reports indicating that the voiding interval is directly correlated with the levels of adducts seen in the bladder following intravesicular treatment (Kadlubar et al., 1991). Adduct levels increased with increasing dose and reached steady state after approximately 6 weeks of chronic

exposure. The adduct levels at steady state appeared to reflect the levels in the bladder urothelium when the animals were sacrificed. These data indicated that this approach might prove to be feasible for human studies.

3. Environmental studies

Tobacco smoke is the most important cause of urinary bladder cancer in the population. Tobacco smokers are thought to be at least a 2-fold increased risk for bladder cancer (IARC, 1986). We have also reported earlier the results of a study of 47 smokers and controls from whom exfoliated urothelial cells were obtained for carcinogen-DNA adduct analysis (Talaska et al., 1991b, 1992b; Vineis et al., 1994). We saw that there were increased levels of carcinogen-DNA adducts in smokers over non-smokers, but that this increase was not statistically significant because of the wide variability, especially in samples later identified as controls. However, the data from the smokers alone was examined, positive correlations were noted between specific DNA adducts in exfoliated urothelial cells, the amount of tobacco smoked, and the levels of mutagens excreted into urine. Most importantly, there was a good correlation between a putative 4-aminobiphenyl-DNA adduct and the corresponding 4-aminobiphenyl-hemoglobin adduct.

4. Occupational studies

The first report of carcinogen-DNA adducts in exfoliated urothelial cells was a case study of a worker with an accidental and massive exposure to methylene-bis-2-chloroaniline (MOCA) (Kaderlik et al., 1993). This study indicated that within 4 h of the accidental exposure extremely high levels of MOCA-DNA adducts were seen in the exfoliated urothelial cells. These levels declined rapidly within 24 h, then declined much more slowly over the next 72 h. The rapid appearance of the adduct in exfoliated cells also suggested that exposure to the active compound occurs intravesicularly and not through the blood because if the latter were true, it would be anticipated that adducts would only slowly ap-

pear as the germinal layer of cells divided and migrated into the lumen.

We have recently presented the results of a study investigating DNA adducts levels in exfoliated urothelial cells in a group of workers exposed to benzidine and benzidine-based dyes (Rothman et al., 1995). We saw that, overall, workers with exposure to benzidine or benzidine-based dyes had levels of adducts nine-fold higher than controls. However, when workers were segregated into benzidine and dye workers a dramatic effect was seen. The average levels of a specific DNA adduct, which shared chromatographic behavior with *N*-(deoxyguanosin-8-yl)-*N'*-acetylbenzidine, were 24 times higher in benzidine production workers than they were in controls ($P \leq 0.001$), while the average levels of the same adduct in the dyeworkers were not significantly elevated over controls. It is noteworthy that the predominate DNA adduct was the acetylated benzidine adduct. This suggests that acetylation is important in the activation of this aromatic bis-amine.

5. Intervention studies

5.1. Used gasoline engine oils

Used gasoline engine oils (UGEO) contain a variety of polycyclic aromatic hydrocarbons (Grimmer et al., 1982, 1983). The published literature indicates that UGEO induce skin cancer in mice treated chronically and carcinogen-DNA adducts are formed in the skin following topical application (Carmicheal et al., 1990). UGEO present a particular problem to an industrial hygiene program because the low volatility of the carcinogenic components and the fact that there is often significant contact with the skin preclude the use of air sampling to predict exposure. For the epidemiologist, auto-mechanics are a difficult population to study because many are probably never exposed to UGEO as they have specialties like tire, brake and air conditioning service. Therefore, the chances of misclassification of persons into an "exposed" group on the basis of job title is high. In addition, most auto shops employ only a few mechanics. Biological markers of effective dose would probably be more valuable under

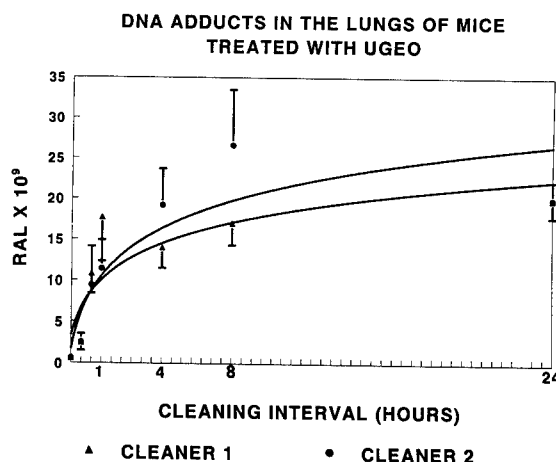


Fig. 2. DNA adducts in the lungs of mice treated topically with used gasoline engine oil. Oils were applied to the shaved skins of groups of animals, then the skins were cleaned at various times with one of two cleaners. The cleaning intervals (in h) are indicated on the x axis. Carcinogen-DNA adduct levels were determined and the total adducts plotted.

these circumstances to reduce exposure misclassification and to measure the effects of intervention strategies. We treated animals with one or five treatments of 50 μ l of UGEO and determined the levels of carcinogen-DNA adducts in the skin, lungs and urinary bladders. We saw that adducts could be measured in each organ following only one treatment with this material and that adduct levels increased with five treatments. We then conducted studies to determine the efficacy of cleaning the skin in a timely manner in reducing the levels of carcinogen-DNA adducts in both the skin and lungs of treated animals. The animals were cleaned at 0.5, 1, 2, 4, or 8 h following application in order to simulate the frequency with which different mechanics might clean their hands. Two cleaning agents were used, one a propylene glycol-based cleaner, the other a petroleum-based cleaner. Figs. 2 and 3 show that when cleaning with either cleaner was performed soon (0.5 or 1 h) after the exposure, carcinogen-DNA adducts from UGEO were significantly reduced. On the other hand, if cleaning was delayed until 8 h after the application, then the levels of carcinogen-DNA adducts were not significantly dif-

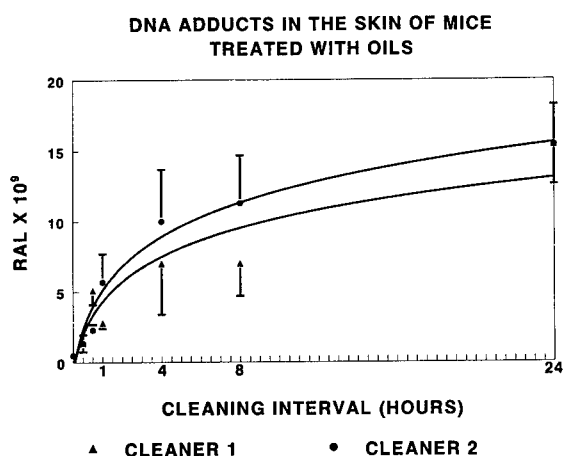


Fig. 3. DNA adducts in the skins of mice treated topically with used gasoline engine oil. Treatment conditions are as given in Fig. 2.

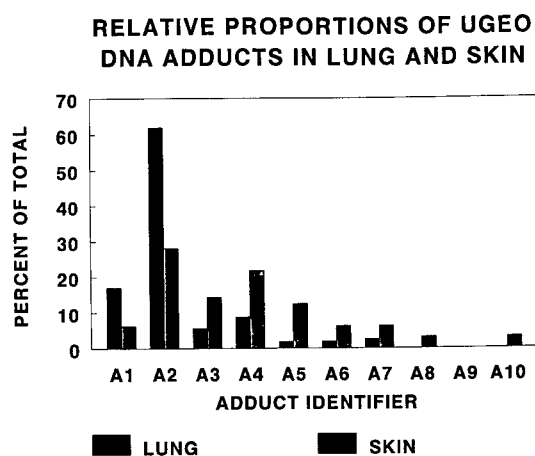


Fig. 4. Relative proportions of used gasoline engine oil-related DNA adducts in lungs and skin of treated animals. The data shown is from animals whose skin was not cleaned following application.

ferent than if cleaning was never done. The same effect was seen in both skin and lung. These data indicate that it is possible to intervene in a carcinogenic exposure and significantly reduce the levels of DNA damage in target organs by some relatively simple measures. Frequent cleaning is currently recommended, but not always practiced in the occupation. These data could be used as a positive reinforcement for frequent cleaning in the workplace.

Fig. 4 displays data for individual adducts by tissue type. This figure displays adduct distribution in lung and skin as a function of the percent of total adducts seen in that tissue. It appears that while the same adducts are seen in both tissues, there are differences in tissue distribution of adducts. This finding suggests that the skin acts as a selective barrier to the passage of certain carcinogens, while others are able to enter the circulation and interact with the distant targets like the lung.

6. Future studies and directions

Since the lung is also a major target for occupational carcinogens we have embarked on a program to utilize cells exfoliated from the lung

and collected in sputum samples for carcinogen-DNA adduct analysis. We have done some exploratory studies which showed that the type and quantity of specific DNA adducts was the same in the cells from sputum and brush biopsies for the same individual.

The incorporation of the use of exfoliated urothelial and bronchial cell techniques and the intervention studies we described above into a comprehensive occupational carcinogen human monitoring program is the future direction of this program. The urinary bladder appears to be a target for the activity of the carcinogens in UGEO. In addition, epidemiological studies have suggested that auto mechanics may be at increased risk for cancer of the urinary bladder. We plan to use non-invasive DNA adduct monitoring techniques as tools to measure the success of interventions with cleaning, barrier creams and combinations thereof in populations of auto mechanics exposed to UGEO.

Other populations of interest for studies would include persons exposed to jet and diesel exhausts, both of which contain significant concentrations of polycyclic aromatic hydrocarbons (PAH) and nitro-PAH. These latter compounds are related to the aromatic amines in that they are reduced to the corresponding *N*-hy-

droxyamines by many biological systems (Delclos et al., 1990).

Conclusions

The advent of sensitive techniques to monitor the level of carcinogen-DNA adducts has made possible non-invasive monitoring of target tissues. These techniques can be used in interventions studies. Once the day-to-day variability in response under steady-state conditions is known for groups of individuals, it should be readily possible to determine the number of samples from each person needed to have a precise estimate of the individual effect.

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Toxicology 111 (1996) 213-224

TOXICOLOGY

Possible health risks from low level exposure to beryllium

Arthur W. Stange^{*a}, Duane E. Hilmas^a, Francis J. Furman^b

^aHealth Effects Department, DynCorp of Colorado, Inc., Rocky Flats Environmental Technology Site, Building 122,
P.O. Box 464, Golden, CO 80402-0464, USA

^bOccupational Medicine Department, DynCorp of Colorado, Inc., Rocky Flats Environmental Technology Site,
Golden, CO 80402-0464, USA

Abstract

The first case of chronic beryllium disease (CBD) at the Rocky Flats Environmental Technology Site (Rocky Flats) was diagnosed in a machinist in 1984. Rocky Flats, located 16 miles northwest of Denver, Colorado, is part of the United States Department of Energy (DOE) nuclear weapons complex. Research and development operations using beryllium began at Rocky Flats in 1953, and beryllium production operations began in 1957. Exposures could have occurred during foundry operations, casting, shearing, rolling, cutting, welding, machining, sanding, polishing, assembly, and chemical analysis operations. The Beryllium Health Surveillance Program (BHSP) was established in June 1991 at Rocky Flats to provide health surveillance for beryllium exposed employees using the Lymphocyte Proliferation Test (LPT) to identify sensitized individuals. Of the 29 cases of CBD and 76 cases of beryllium sensitization identified since 1991, several cases appear to have had only minimal opportunistic exposures to beryllium, since they were employed in administrative functions rather than primary beryllium operations. In conjunction with other health surveillance programs, a questionnaire and interview are administered to obtain detailed work and health histories. These histories, along with other data, are utilized to estimate the extent of an individual's exposure. Additional surveillance is in progress to attempt to characterize the possible risks from intermittent or brief exposures to beryllium in the workplace.

Keywords: Beryllium; Health; Surveillance; Sensitization; Exposure

1. Introduction

Chronic beryllium disease (CBD) is a chronic granulomatous disorder of the lungs following the inhalation of beryllium, in which a specific cell-mediated immune response plays a central

role (Jones and Williams, 1983; Rossman et al., 1988; Kreiss et al., 1989). The pathogenesis of CBD is believed to be a cell-mediated hypersensitivity reaction to beryllium bound to tissue proteins. According to the criteria established by the Beryllium Case Registry, a diagnosis of CBD included at least four of the following six conditions with one of the first two conditions required: (1) significant beryllium exposure; (2) the presence of beryllium in lung tissue, lymph nodes

^{*}Corresponding author, Tel.: 303-966-8373; Fax: 303-966-2873.

or urine; (3) evidence of radiologic interstitial lung disease; (4) lower respiratory tract disease with a clinical course consistent with CBD; (5) obstructive or restrictive ventilatory defects or decreased carbon monoxide diffusing capacity; and (6) non-caseating granulomas on lung biopsy (Hardy, 1957; Hardy et al., 1967). Currently, hypersensitivity to beryllium as demonstrated through the lymphocyte proliferation test (LPT) is required as one of the criteria for the diagnosis of CBD (Jones and Williams 1983; Rossman et al., 1988; Kreiss et al., 1989).

In 1949 the United States Atomic Energy Commission (AEC) implemented a beryllium exposure standard of $2 \mu\text{g}/\text{m}^3$ of air as a time-weighted average (TWA) and a ceiling of $25 \mu\text{g}/\text{m}^3$ based on investigations of known carcinogenic metals (EPA, 1987). The Occupational Safety and Health Administration (OSHA) adopted an 8-h TWA of $2 \mu\text{g}/\text{m}^3$ for beryllium. The American Conference of Government Industrial Hygienists (ACGIH) established $2 \mu\text{g}/\text{m}^3$ as the Threshold Limit Value (TLV) for beryllium. The National Institute for Occupational Safety and Health established an evaluation criterion level of $0.5 \mu\text{g}/\text{m}^3$ for beryllium exposure and a TWA of $2 \mu\text{g}/\text{m}^3$ (NIOSH, 1972).

The decreasing number of CBD cases reported to the Beryllium Disease Case Registry in the 1970s seemed to indicate that the $2 \mu\text{g}/\text{m}^3$ exposure limit had been effective (Sprince and Kazemi, 1980). However, a high percentage (2%) of the cases of occupational CBD had been diagnosed in secretaries employed in beryllium plants or laboratories who should have received minimal exposure to beryllium (Eisenbud and Lisson, 1983).

Since 1957 when beryllium production operations began at Rocky Flats, 39 of 72 production buildings have contained some type of beryllium operation, although one building contained the majority of these operations. Beryllium production operations consisted of foundry and casting, shearing, rolling, cutting, welding, machining, sanding, polishing, assembly, and chemical analysis. The potential for occupational exposure to beryllium occurred to varying degrees in these 39 buildings.

From 1957 through 1984 the primary method for beryllium exposure assessment at Rocky Flats was fixed airhead sampling. Fixed airhead sampling filters were attached to beryllium production machines to provide air sampling within a few feet of the worker's breathing zone. In addition, fixed airhead sampling filters were randomly positioned in areas that contained beryllium operations. Personal breathing zone sampling was employed to monitor beryllium work processes following the diagnosis of the first case of CBD at Rocky Flats in 1984.

Investigations of a stratified population of Rocky Flats employees from 1987 through 1991 have led to the identification of 23 cases of CBD and an additional three cases of beryllium sensitization (Kreiss et al., 1993). The identification of CBD in an employee who had no identifiable contact with beryllium raised the concern that low level beryllium exposure might lead to beryllium sensitization and CBD.

This paper describes an ongoing program to identify and assess beryllium sensitization and CBD in current and former Rocky Flats employees. The BHSP uses the LPT for the detection of beryllium sensitization, B-reader evaluated posterior/anterior chest X-rays to identify individuals with radiographic findings possibly related to CBD, and medical evaluations for CBD at two major medical centers experienced in diagnosing CBD. The BHSP has demonstrated the need for periodic beryllium sensitivity retesting for individuals who previously tested LPT negative, and has identified cases of beryllium sensitization and CBD in individuals that appear to have had only minimal opportunistic exposures to beryllium.

2. Methods

2.1. Beryllium exposure assessment at Rocky Flats

A hierarchical system of job and building exposure factors was modified to provide relative estimates of beryllium exposure (Viet and Torma-Krajewski, 1995). Job factors (0 through 10) were assigned to jobs held by beryllium-sensitized and CBD cases by Rocky Flats industrial hygienists, based on the potential for beryl-

lium exposure relative to a job factor of 10 for a beryllium machinist. When an individual worked more than one job, the job with the greatest factor was used to designate their job factor as long as the individual was in that job for a minimum of one month. Building factors (0 through 10) were assigned for all buildings by Rocky Flats industrial hygienists based on the potential for beryllium exposure relative to a factor of 10 for the main beryllium production building. When an individual worked in several buildings, the building with the greatest factor was used to designate their building factor as long as the individual worked in that building for a minimum of one month. Job and building factors were given equal weight in determining a composite beryllium exposure factor for each beryllium-sensitized and CBD case. The composite beryllium exposure factor was obtained by multiplying the job and building factors. As the relationship between the level of beryllium exposure required and the length of time/frequency of beryllium exposure which results in beryllium sensitization is not known, this method was chosen for estimating relative exposure among the CBD and beryllium-sensitized population. Job titles and buildings where work was performed by beryllium sensitized or CBD cases were obtained from personnel records, industrial hygiene personal breathing zone sampling records, medical records, and from completed BHSP questionnaires.

A review of historical personal breathing zone beryllium sampling data revealed a very limited amount of sampling data was ever collected for individuals sensitized to beryllium or diagnosed with CBD. As a result, no attempt was made in this investigation to assign potential beryllium exposure values to either the building or job exposure factors for the sensitized or CBD populations. General assumptions regarding the relative potential for exposures to beryllium for jobs and buildings will be discussed.

To further assess the potential for beryllium exposure in workers at Rocky Flats, a random sample of fixed airhead samples was selected from the main beryllium production building (Barnard and Torma-Krajewski, 1994). The main

beryllium production building was chosen as it had the highest potential for employee exposures to beryllium over the longest period of time, and a wide variety of beryllium production operations occurred in this building between 1957 and 1993. In addition, beryllium foundry and casting operations took place in this building from 1965 through 1975. Over half of the fixed airhead samples collected at Rocky Flats between 1957 and 1993 were from this building. The random sample ($n = 7455$) was obtained from fixed airhead samples from two beryllium production rooms within the main beryllium production building. The sample consisted of two fixed airhead samples per month per machine for 18 beryllium production machines for the period 1970 through 1988.

Fixed airhead beryllium samples ($n = 102$) and personal breathing zone beryllium samples ($n = 102$) from the main beryllium production building were matched by production machine number for January 1984 through February 1986. A matched pairs analysis was used to determine if there was any relationship between fixed airhead beryllium samples and personal breathing zone beryllium samples (Barnard and Torma-Krajewski, 1994).

2.2. Surveillance population

Twenty-three cases of CBD have been diagnosed from a cohort of Rocky Flats employees ($n = 954$) tested prior to the implementation of the BHSP in June, 1991. These prior investigations had focused on employees with job descriptions that had been identified as having known beryllium exposure potential, although one case of CBD was diagnosed in an employee who had no identifiable contact with beryllium (Kreiss et al., 1993). As the result of this CBD case, and after initial attempts of identifying employees with the potential for beryllium exposure at any level proved difficult, it was decided to allow employees of Rocky Flats to self-identify as having had the potential for exposure to beryllium and to participate in the BHSP.

2.3. Lymphocyte proliferation test (LPT)

The peripheral blood lymphocyte proliferation

test (LPT) which measures lymphocyte proliferation in response to beryllium salts (beryllium sulfate, beryllium fluoride) in vitro is elevated in individuals with CBD and in those sensitized to beryllium (Jones and Williams, 1983; Rossman et al., 1988; Kreiss et al., 1989). This lymphocyte proliferation in response to beryllium salts was demonstrated by Hanifin et al. (1970), and by Deodhar et al. (1973). Current data suggest that this proliferative response is a specific and sensitive method for testing for beryllium sensitization (Jones and Williams, 1983; Rossman et al., 1988). The use of bronchoalveolar lavage lymphocytes for the LPT has aided the diagnosis of CBD (Rossman et al., 1988).

The peripheral blood LPT was used to identify beryllium-sensitized individuals in the BHSP. Three laboratories with recognized expertise in performing beryllium LPTs analyzed submitted peripheral blood specimens. An individual was considered beryllium sensitized when two consecutive peripheral blood LPTs were found to be positive from one LPT laboratory or any two LPT laboratories in combination. If an individual was found to have a positive peripheral blood LPT on a single test, they received a second blood draw. Blood specimens were sent to two LPT laboratories to confirm the positive LPT. If the second LPT was found to be positive at either laboratory, the individual was listed as sensitized to beryllium. An individual was also listed as sensitized to beryllium if the bronchoalveolar lavage LPT was positive, but the peripheral blood LPT was negative.

2.4. Beryllium health surveillance program administration

The BHSP is a voluntary participation program offered to all current and former employees of prime contractors, subcontractors, DOE, and various temporary Crafts and Trades Union employees of Rocky Flats. An LPT Informed Consent and BHSP Questionnaire Informed Consent were developed with input from the various participant groups, reviewed by the Rocky Flats Human Subjects Review Committee, and approved for use by the Legal Departments of Rocky Flats and the DOE.

Each BHSP participant provided a signed BHSP Questionnaire Informed Consent, a signed LPT Informed Consent, and a completed self-administered BHSP questionnaire containing medical and occupational history questions. Participants received a venipuncture with 30 ml of blood collected for the peripheral blood LPT (60-90 ml collected for quality control specimens), and a posterior/anterior chest X-ray for B-reader review.

2.5. Chest X-rays

Posterior/anterior chest X-rays were evaluated according to the International Labor Organization (ILO) classification system for radiographs of pneumoconioses by board certified radiologists who were certified B-readers (International Labor Organization, 1980). The presence of noncaseating granulomas and/or mononuclear infiltrates is consistent with CBD (Freiman and Hardy, 1970). Abnormal profusion of small opacities was defined as profusion greater than or equal to 1/0. Chest X-rays submitted for B-reader evaluation were returned to the Rocky Flats Medical Director who scored each from the information provided by the B-reader radiologist(s) according to the following scheme: (1) no abnormality; (2) an abnormality not associated with CBD, profusion 0/1 or less; (3) an abnormality possibly associated with CBD, profusion 1/0 or greater. Participants with a chest X-ray abnormality possibly associated with CBD, profusion 1/0 or greater, were contacted and informed of the availability of additional diagnostic procedures for a determination of CBD, and subsequently referred for a CBD medical evaluation.

2.6. Medical evaluations for CBD

All beryllium-sensitized individuals and all individuals with a small opacity profusion of 1/0 or greater on chest X-ray were offered a thorough medical evaluation at one of two major medical facilities in the United States with experience in the diagnosis of CBD. Referrals for CBD medical evaluations were made for any of the following reasons: (1) beryllium-sensitized — peripheral blood LPT positive on two separate test dates or

one test date by two laboratories; (2) borderline peripheral blood LPT positive on three test dates; (3) single peripheral blood LPT positive (positive only on one day at one beryllium concentration) at one or more laboratories on three test dates; (4) chest X-ray with small opacity profusion of 1/0 or greater; and (5) clinical symptoms consistent with CBD (results from peripheral blood LPT and chest X-ray previously found negative).

Following the identification of beryllium sensitization, a diagnosis of CBD in the BHSP required a positive bronchoalveolar lavage LPT, and histologic evidence of pulmonary granulomatous disease. CBD was diagnosed without evidence of peripheral blood sensitization (two positive peripheral blood LPTs) if a positive bronchoalveolar lavage LPT result was obtained, and there was histologic evidence on biopsy of pulmonary granulomatous disease. A probable diagnosis of CBD includes a history of exposure to beryllium or relevant documentation of working in a building or area with the potential for beryllium exposure and two of the following: (1) beryllium sensitization as measured by the peripheral blood LPT; (2) a positive bronchoalveolar lavage LPT; or (3) evidence of granulomatous disease by chest X-ray or CT scan.

2.7. Three-year/one-year beryllium health surveillance program retesting

In August 1993, the DOE approved LPT retesting for those current Rocky Flats employees who had not been tested for 3 years or more and who previously tested LPT negative. Current and former employees were also offered retesting if they had previously received an unconfirmed positive LPT result. Current and former employees with a profusion of 0/1 were offered a 1-year LPT retest and chest X-ray to determine if changes occurred in their LPT results or in their profusion of small opacities. All individuals with a profusion of small opacities of 1/0 or greater who completed a clinical evaluation for CBD, but for whom no definitive diagnosis was reached, were offered a 1-year LPT retest and chest X-ray. Periodic LPT retesting allows for the

identification of LPT negative to positive conversions, and also identifies false negative LPT results.

Current and former Rocky Flats employees who had previously participated in the BHSP were contacted by telephone and offered 1-year or 3-year retesting. Contacting previous participants by telephone allowed individuals to ask questions regarding the BHSP in general, and to ask questions concerning the 1-year and 3-year resting phase of the program. It was believed that telephone contact with participants, as opposed to contact through the mail, would provide individuals with an avenue to voice concerns regarding beryllium sensitization and CBD, and to voice their opinion regarding their participation in the BHSP.

3. Results

3.1. CBD and beryllium sensitization cases

Table 1 shows the characteristics of the BHSP surveillance population tested between June 1, 1991 and March 31, 1995. During this period 1885 current Rocky Flats employees received their initial LPT as a participant in the BHSP. Six cases of CBD have been diagnosed in current employees, and 22 current employees have been identified as beryllium-sensitized as the result of double positive LPTs. During the same time period, 2512 former Rocky Flats employees received an LPT as a participant in the BHSP. Of this group, 22 individuals have been diagnosed with CBD, and 47 former employees have been identified as beryllium-sensitized as the result of repeat positive LPTs. Individuals identified as sensitized have diagnostic evaluations for CBD in progress. The total sensitization rate (CBD and sensitized cases) for the tested cohort ($n = 4397$) was 2.21%. The CBD rate was 0.64% and the beryllium sensitization rate was 1.57%.

Three-year/one-year LPT retesting has resulted in the identification of one case of CBD and nine cases of beryllium sensitization out of the 518 current and former employees retested (Table 1). The total sensitization rate (CBD and sensitized cases) for the three-year/one-year retested cohort of 1.93% is not statistically different from

Table 1
Beryllium health surveillance population (BHSP)

	Initial LPT	1-Year/ 3-year LPT
Current Rocky Flats employees		
Number contacted	8772	512
Participation forms returned	3902	n/a
Number requesting participation	1932	452
BHSP participation completed	1885	452
Outcome		
CBD	6	1
LPT positive (sensitized)	22	7
CBD and sensitization percentage	1.49	1.77
Former Rocky Flats employees		
Number contacted	9865	74
Participation forms returned	3602	n/a
Number requesting participation	2826	66
BHSP participation completed	2512	66
Outcome		
CBD	22	0
LPT positive (sensitized)	47	2
CBD and sensitization percentage	2.75	3.03
Current and former employee CBD and sensitization percentage	2.21	1.93

the rate observed from initial LPT findings. After completing CBD medical evaluations, one of these individuals was diagnosed with CBD, and the nine remaining sensitized individuals will be offered annual CBD medical evaluations.

Of the 29 cases of CBD identified, seven cases had no evidence of granulomas on biopsy, and no biopsy was performed for another five cases as the result of complicating medical conditions. These 12 cases were categorized as probable cases of CBD rather than confirmed cases of CBD.

Of the 518 individuals (452 current employees, 66 former employees) who participated in one-year/three-year retesting, nine had a prior borderline or positive LPT, 34 had a chest X-ray with a opacity profusion rating of 1/0 or greater after B-reader evaluation, and 475 had a prior negative LPT findings. Of the ten individuals who were identified as sensitized after LPT retesting, nine of these individuals had prior nega-

tive LPT findings. The remaining individual had a prior borderline positive LPT that was not confirmed as positive in initial testing.

Of the B-reader reviewed chest X-rays for the 29 individuals diagnosed with CBD, only one case had a small opacity profusion of 1/0 or greater, one had a small opacity profusion of 0/1, and the remaining 27 were rated as 0/0. For the 78 beryllium-sensitized cases, B-reader chest X-ray results revealed three cases with small opacity profusion of 1/0, one case with a small opacity profusion of 0/1, and the remaining 74 cases were rated as 0/0. To date, only one case of CBD has been identified where chest X-ray findings were indicative of CBD not already suggested by the peripheral blood LPT.

Current employees of Rocky Flats who were identified as sensitized to beryllium or who were diagnosed with CBD were notified of the potential hazards related to further exposure to beryllium at any level. Employees with CBD were transferred to work areas where there was no potential for exposure to beryllium. Individuals who are sensitized to beryllium are offered medical evaluations on an annual basis to monitor for the development of CBD. Individuals diagnosed with CBD are offered medical evaluations on an annual basis to monitor the progression of the disease, and to provide treatment where appropriate.

3.2. Beryllium exposure assessment at Rocky Flats

Table 2 shows the building, job, and composite beryllium exposure factors that were assigned to the CBD cases, and Table 3 shows the building, job, and composite beryllium exposure factors that were assigned to the beryllium-sensitized cases. Beryllium sensitization and CBD cases occurred in a wide variety of job and building exposure categories at Rocky Flats, and were diagnosed in occupational groups previously demonstrated susceptible in an earlier beryllium health study at Rocky Flats (Kreiss et al., 1993). From these data it is apparent that a wide range of potential beryllium exposures occurred in these populations. Several cases of CBD and beryllium sensitization were identified in jobs that were considered to have had only minimal

Table 2
Current and former Rocky Flats chronic beryllium disease cases

Job title(s)	Beryllium exposure factors		
	Building factor	Job factor	Composite factor
<i>Current cases</i>			
Custodian, metallurgical operator	10	10	100
Air filter technician, electrician	10	8	80
Electrician, electrical technician	10	8	80
Sheet metal worker	10	7	70
Labor pool, radiation protection technician	10	5	50
Janitor, decontamination, clerk packer, machine operator, modification technician	10	4	40
Radiation protection technician, health physics technician, safety committee, safety technical advisor	8	5	40
<i>Former cases</i>			
Machinist	10	10	100
Machinist	10	10	100
Machinist, maintenance machinist	10	10	100
Machinist, tool maker	10	10	100
Labor pool, chemical operator, metallurgical operator	10	10	100
Electrician, planner, foreman, maintenance supervisor	10	8	80
Instrument repair, electrical technician, electrician	10	8	80
Janitor, equipment operator, sheet metal worker	10	7	70
Sheet metal worker	8	7	56
Toolmaker, inspector	10	5	50
Janitor, radiation monitor, foreman health physics	10	5	50
Janitor, chemical operator, machinist apprentice	10	4	40
Labor pool, machinist apprentice, truck driver	10	4	40
Laborer, janitor	10	3	30
Chemical engineer	7	2	14
Engineer	10	1	10
Stenographer, secretary, administrative support	10	1	10
Secretary	8	1	8
Chemical operator, waste treatment, maintenance electrician	1	8	8
Laboratory technician	3	2	6
Chemical operator	1	3	3
Design engineer	2	1	2

opportunistic exposures to beryllium since these individuals were employed in administrative functions rather than primary beryllium operations.

More than 500 000 fixed airhead samples were collected from 1957 through 1993, and from 1957 through 1984 served as the primary method for a general characterization of employee exposure(s) to beryllium. Fixed airhead samples were collected on a daily basis from production machines to provide beryllium monitoring within a few feet

of the worker's breathing zone. To monitor ambient beryllium levels in rooms that contained beryllium operations industrial hygienists and production engineers randomly positioned additional fixed airhead filters. Beginning in 1984 after the identification of the first case of CBD in a beryllium machinist, breathing zone sampling was used to monitor changes in beryllium levels as the result of changes in beryllium work processes and procedures, as well as changes in beryllium production equipment. However, as a

Table 3
Current and former Rocky Flats beryllium-sensitized cases

Job title(s)	Beryllium exposure factors		
	Building factor	Job factor	Composite factor
<i>Current cases</i>			
Machinist, foreman machining, security	10	10	100
Machinist, tool maker	10	10	100
Equipment operator, decontamination foreman	10	10	100
Janitor, electrician, maintenance	10	8	80
Inspector, maintenance machinist	10	7	70
Tool grinder, quality engineer	10	7	70
Stationary operating engineer, laundry worker	10	7	70
Pipefitter, modification machinist	10	6	60
Pipefitter, maintenance	10	6	60
Clerk packer, sheet metal worker	8	7	56
Quality assurance inspector	10	5	50
Quality assurance specialist	10	5	50
Janitor, decontamination, pipefitter	7	6	42
Janitor, lubrication worker	10	4	40
Tool maker	10	4	40
Tool maker, program engineer, developmental engineer	10	4	40
Chemical operator, radiation monitor, laboratory technician, waste technician	7	5	35
Laborer, clerk packer, material handler, non-destructive test technician	10	3	30
Metallurgical engineer	10	3	30
Laborer, radiation monitor	5	5	25
Chemical operator, program management	7	3	21
Engineer	10	1	10
Engineer	10	1	10
Labor pool, modification center	10	1	10
Standards engineer, tool and gage engineer, manufacturing engineer, facilities quality engineer	3	2	6
Laboratory technician	2	3	6
Pipefitter	1	6	6
Labor pool, janitor, chemical operator, radiation monitor	1	5	5
Carpenter	3	1	3
<i>Former cases</i>			
Machinist	10	10	100
Machinist	10	10	100
Maintenance machinist, toolmaker apprentice	10	10	100
Machinist, supervisor machining	10	10	100
Janitor, Radiographer, experimental operator	10	10	100
Chemical operator, experimental operator	10	10	100
Sheet metal worker	10	10	100
Sheet metal worker	10	10	100
Electrician	10	8	80
Maintenance electrician	10	8	80
Janitor, chemical operator, metallurgical operator, engineer	8	10	80
Janitor, clerk packer, sheet metal worker	10	7	70
Sheet metal worker	10	7	70
Electrician	8	8	64
Parts inspector, tool and gage inspector	10	5	50

Table 3 (Continued)

Job title(s)	Beryllium exposure factors		
	Building factor	Job factor	Composite factor
Guard, inspector, procurement	10	5	50
Laundry, maintenance, inspector	10	5	50
Janitor	10	4	40
Janitor, clerk packer, material analyst, radiation protection technician	7	5	35
Janitor, assembler	8	4	32
Metallurgical analyst, foreman, supervisory engineer	10	3	30
Metallurgist, research specialist	10	3	30
Maintenance pipefitter	5	6	30
Waste process engineer, waste management, technology support engineer	7	3	21
Sheet metal worker, construction	3	7	21
Chemist, foreman, engineer	10	2	20
Laborer, laboratory technician, chemist	10	2	20
Cooperative engineer	10	2	20
Laboratory technician	8	2	16
Guard, fire-fighter	5	3	15
Design technician	10	1	10
Clerk, secretary	10	1	10
Plutonium laboratory, waste management	5	2	10
Chemical operator	3	3	9
Secretary	8	1	8
Heating and air-conditioning operator, boiler operator	8	1	8
Cost accountant, auditor	8	1	8
Key punch operator	8	1	8
Pipefitter	1	6	6
Pipefitter, welder, sheet metal worker	1	6	6
Clerk, secretary	5	1	5
Clerk	5	1	5
Laborer, warehouse clerk	1	1	1
Manufacturing engineer, production engineer	1	1	1
Engineer	0	1	0
Purchasing manager	0	1	0
Personnel manager	0	0	0
Administrative support	0	0	0
Production technician	0	0	0

result of this sampling scheme a limited number of personal breathing zone beryllium samples were collected for the majority of CBD and beryllium-sensitized cases. Personal breathing zone sampling from 1984 through 1993 yielded more than 1700 samples.

The data in Table 4 show the number of samples and the mean concentration of beryllium obtained from the random sample of fixed airhead beryllium samples for the years 1970

through 1988. These data from two beryllium production rooms of the main beryllium production building indicate that exposure levels were below the established $2.0 \mu\text{g}/\text{m}^3$ TLV for beryllium. Table 4 also shows the number of personal breathing zone beryllium samples and the mean concentration of beryllium for January 1984 through February 1986. Analysis was performed on 102 matched fixed airhead and personal breathing zone beryllium samples. These data

Table 4
Beryllium sampling: main beryllium production building 1970–1988

Year	Fixed airhead		Personal	
	Number of samples (random sample)	Mean ($\mu\text{g}/\text{m}^3$)	Number of samples	Mean ($\mu\text{g}/\text{m}^3$)
1970	308	0.31	—	—
1971	402	0.36	—	—
1972	430	0.36	—	—
1973	430	0.42	—	—
1974	416	0.23	—	—
1975	432	0.16	—	—
1976	431	0.11	—	—
1977	432	0.12	—	—
1978	431	0.13	—	—
1979	369	0.10	—	—
1980	410	0.16	—	—
1981	426	0.14	—	—
1982	432	0.16	—	—
1983	432	0.27	—	—
1984	423	0.23	33	1.09
1985	396	0.16	51	1.20
1986	290	0.18	62	0.46
1987	255	0.03	16	0.19
1988	310	0.05	—	—

also support the fact that following the identification of the first CBD case, and the installation of a new air filtration system in the main production building in 1986, beryllium exposure levels declined as measured by fixed airhead and personal breathing zone sampling. The mean concentration of beryllium from fixed airhead data was $0.16 \mu\text{g}/\text{m}^3$ (standard deviation = 0.33) with a 95% confidence interval of 0.10–0.22 $\mu\text{g}/\text{m}^3$, and the mean exposure level to beryllium from breathing zone data was $1.04 \mu\text{g}/\text{m}^3$ (standard deviation = 1.25) with a 95% confidence interval of 0.79–1.29 $\mu\text{g}/\text{m}^3$. There was no correlation ($r^2 = 0.029$) between fixed airhead and personal breathing zone beryllium samples (Barnard, 1994).

4. Discussion

The BHSP participants tested from June 1991 through March 1995 ($n = 4397$) were comprised

of 42.8% current employees and 57.2% former employees of Rocky Flats. Nearly fifty percent (49.5%) of current Rocky Flats employees requested participation in the BHSP, and participation was requested by 78.5% of former Rocky Flats employees. Based upon these rates, it appears that former employees had an increased level of concern regarding exposure to beryllium and related health effects. However, this increased participation rate might also have been because former employees had not previously had the same opportunity to receive testing for beryllium sensitivity as current employees.

The sensitization rate (CBD and sensitized cases) for the population that received both initial LPT and one-year/three-year LPT retesting ($n = 4397$) was 2.43%. This rate is comparable to the sensitization rate for a previously studied beryllium-exposed population ($n = 954$) at Rocky Flats which was 2.5% (Kreiss et al., 1993). It is important to note the previous study population was a stratified random sample of current

Rocky Flats employees selected based upon a significant opportunity for exposure to beryllium, and the current BHSP study population is comprised of current and former employees with any level of exposure to beryllium.

The usefulness of the peripheral blood LPT on a routine basis to monitor a beryllium-exposed population for the development of beryllium sensitization and CBD was demonstrated in the BHSP study population. Because of the potential for false positive LPT results, a beryllium-sensitized individual is identified as one in whom two consecutive positive LPTs have been found. Retesting to confirm initial positive LPTs greatly diminishes the likelihood of falsely identifying individuals as being beryllium-sensitized when they are not.

The overall value of posterior/anterior chest X-rays evaluated by B-reader radiologists as a means for the identification of CBD appears limited. The chest X-ray is insensitive and non-specific for the diagnosis of CBD. Based upon these data, the posterior/anterior chest X-ray should not be used as the sole means of screening for CBD and/or beryllium sensitization.

Job titles and buildings where work was performed by CBD cases and by individuals identified as beryllium-sensitized were obtained from personnel records, industrial hygiene records, medical records, and from completed BHSP questionnaires. An evaluation of the job and building factors of the beryllium-sensitized and CBD cohorts suggests that exposure to beryllium at levels below the TLV of $2.0 \mu\text{g}/\text{m}^3$ may result in beryllium sensitization. This may be particularly important for individuals who may be more sensitive to beryllium exposures than the general population (Richeldi et al., 1993). Job and building exposure factors assigned by the Rocky Flats Industrial Hygiene Department were given equal weight in creating composite factors. As the relationship between the level of beryllium exposure required and the length of time/frequency of beryllium exposure which results in beryllium sensitization is not known, this method for estimating relative exposure among the CBD and beryllium-sensitized populations seems appropriate. The long latency period for the development of beryl-

lium sensitization and/or CBD and the potential for continued low level exposures over time makes the determination of a safe level difficult. It is possible that many of the beryllium sensitization cases with limited potential for exposure to beryllium may have resulted from a significant number of excursions above the TLV of $2.0 \mu\text{g}/\text{m}^3$ which were not detected, or cases of sensitization occurred at levels significantly below the TLV.

No attempt was made to assign beryllium exposure values to either the building or job exposure factors for the sensitized or CBD populations as personal breathing zone sampling data was never collected for the majority of these individuals. In general the relative potential for exposures to beryllium was the greatest for employees in machining and metallurgical operations, and the least for administrative positions. Of note was the high relative exposure potential for jobs in maintenance, and a variety of crafts and trades. Cases of beryllium sensitization and CBD were found in jobs with composite exposure factors ranging from 100 to 0. This demonstrates the need for ongoing beryllium health surveillance for employees in all jobs that have the potential for exposure to beryllium.

The need for LPT retesting has been demonstrated through the identification of ten cases of beryllium sensitization in individuals who previously tested normal ($n = 518$). These ten individuals between 1989 and 1991 had negative peripheral blood LPT results. One of these individuals, following a medical evaluation, has been diagnosed with CBD. Although there is no standard for retesting, we have established a 3-year retest frequency for individuals who previously were negative on the peripheral blood LPT. More frequent retesting, for example annually, may be recommended if prior clinical findings or recent symptoms suggest CBD. These ten LPT retest cases represent either of the following situations: (1) LPT negative to positive conversions; (2) the identification of prior false negative LPT results.

As the use of beryllium continues to increase in aerospace, electronics, and a variety of other industries, the health surveillance of beryllium-exposed populations becomes vitally important

to occupational physicians and to epidemiologists in occupational settings. Occupational physicians in industries that use beryllium and/or beryllium alloys should strongly consider the potential for employees to develop beryllium sensitization and CBD even though routine health monitoring is provided. It is important to recognize that for some employees exposure to beryllium at levels below the TLV might cause disease. We have reported on a population of employees where applicable safety exposure requirements for beryllium were maintained yet beryllium sensitization and CBD occurred. We have also reported cases of sensitization and CBD in employees with jobs that were thought not to be of concern because of their minimal opportunity for exposure to beryllium.

The Rocky Flats BHSP is a health surveillance program conducted by an industrial Occupational Medicine department in which cases of beryllium sensitization and CBD are identified and tracked in current and former employees who have been occupationally exposed to beryllium. The BHSP has provided and will continue to provide important information for occupational physicians and private health care professionals concerning the design, implementation, and ongoing management of a program for the detection of beryllium sensitization and CBD in populations at risk.

Acknowledgements

This work was conducted by the EG & G Rocky Flats, Inc., Rocky Flats Environmental Technology Site, Health Effects Department with funding from the United States Department of Energy under Operating Contract DE-AC04-90DP62344.

The authors wish to thank D.M. Splett for providing statistical support for the Rocky Flats Beryllium Health Surveillance Program, and to thank C.L. Mathews and M.E. McCoy for assisting with manuscript preparation.

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ELSEVIER

Toxicology 111 (1996) 225-238

TOXICOLOGY

Sampling and analysis of airborne resin acids and solvent-soluble material derived from heated colophony (rosin) flux: a method to quantify exposure to sensitizing compounds liberated during electronics soldering

Philip A. Smith^a, Paul S. Son^{*b}, Patrick M. Callaghan^b, Warren W. Jederberg^b,
Karl Kuhlmann^c, Kenneth R. Still^b

^aNaval Hospital Jacksonville, Jacksonville, FL, USA

^bNaval Medical Research Institute Detachment (Toxicology), Wright-Patterson Air Force Base, Dayton, OH 45433, USA

^cManTech Environmental Technology, Inc., P.O. Box 31009, Dayton, OH 45437, USA

Abstract

Components of colophony (rosin) resin acids are sensitizers through dermal and pulmonary exposure to heated and unheated material. Significant work in the literature identifies specific resin acids and their oxidation products as sensitizers. Pulmonary exposure to colophony sensitizers has been estimated indirectly through formaldehyde exposure. To assess pulmonary sensitization from airborne resin acids, direct measurement is desired, as the degree to which aldehyde exposure correlates with that of resin acids during colophony heating is undefined. Any analytical method proposed should be applicable to a range of compounds and should also identify specific compounds present in a breathing zone sample. This work adapts OSHA Sampling and Analytical Method 58, which is designed to provide airborne concentration data for coal tar pitch volatile solids by air filtration through a glass fiber filter, solvent extraction of the filter, and gravimetric analysis of the non-volatile extract residue. In addition to data regarding total soluble material captured, a portion of the extract may be subjected to compound-specific analysis. Levels of soluble solids found during personal breathing zone sampling during electronics soldering in a Naval Aviation Depot ranged from below the "reliable quantitation limit" reported in the method to 7.98 mg/m³. Colophony-spiked filters analyzed in accordance with the method (modified) produced a limit of detection for total solvent-soluble colophony solids of 10 µg/filter. High performance liquid chromatography was used to identify abietic acid present in a breathing zone sample.

Keywords: Airborne; Analysis; Colophony; Gravimetric; Rosin; Sensitization

*Corresponding author.

1. Introduction

Colophony (rosin) is a natural product derived from pine resin with wide applications in industrial processes, including use in many commercial fluxes and use as "rosin core" solder in electronics soldering (Enos et al., 1968; American Conference of Governmental Industrial Hygienists, 1992). The chemical constituents and their proportions in colophony are species dependent and vary widely, depending not only upon the pine resin source, but also on the method of extraction (Joy and Lawrence, 1967). In terms of gross chemical makeup, rosin has been reported to consist of about 90% resin acids and 10% neutral matter, with about 90% of the resin fraction being isomeric forms of abietic acid ($C_{20}H_{30}O_2$), and the remaining 10% a mixture of dihydroabietic acid ($C_{20}H_{32}O_2$) and dehydroabietic acid ($C_{20}H_{28}O_2$) (Enos et al., 1968; The Merck Index, 1983c). Levopimaric, pimaric, palustric, neoabietic acid and other isomers of these acids have been reported as rosin fraction constituents (Enos et al., 1968; Joy and Lawrence, 1967). The neutral fraction reportedly contains stilbene and terpene derivatives, aldehydes, and several hydrophenanthrene-based hydrocarbons (Enos et al., 1968). Rosin is a solid at room temperature. Joy and Lawrence (1967) reported the melting point range for 18 specific resin acid compounds to be 24–219°C, with the melting point for abietic acid (a common resin acid constituent) at 172–175°C.

Airborne material termed "rosin smoke" consists of respirable particles (Hatch and Gross, 1964). Fawcett et al. (1976) described asthmatic reactions to solder fume exposures attributed to airborne flux material. A number of subsequent studies identified respiratory effects among electronics industry workers with occupational exposure to heated airborne rosin flux. (Burge et al., 1978, 1979a,b, 1980; Perks et al., 1979; Burge, 1982a,b; Reilly et al., 1994). Two of these studies included bronchial provocation challenges showing 'positive' responses to either heated rosin core solder or heated colophony alone, and "non-sensitized" workers showing no significant reaction (Burge et al., 1978, 1980). Another study

identified respiratory effects in a factory where rosin was heated to manufacture "flux-cored" solder (Burge et al., 1981). In the preceding studies of occupational asthma related to airborne colophony contaminants, the specific airborne compounds which caused the pulmonary sensitization were not identified.

Gas and liquid chromatography have been applied to identify low molecular weight carboxylic acids and aldehydes derived from heated rosin (Drugov and Murav'eva, 1976; Guenier et al., 1984), but the full range of airborne compounds derived from heated flux has not been established (Burge, 1984; Lesage and Perrault, 1993).

Several literature citations recognize exposure during soldering with rosin flux as a potential source of contact allergy (Widstrom, 1983; Mathias and Adams, 1984; Goh and Ng, 1987). A large volume of literature reports that dermal exposures to natural and modified resin acids are associated with contact allergy. Several citations offer thorough reviews of rosin-associated contact allergy (Karlberg, 1988; Hausen et al., 1989).

Abundant data show airborne contaminants from heated rosin to be pulmonary sensitizers, and resin acids found in rosin to be skin sensitizers. In spite of this, no work has been performed to characterize the types and levels of airborne resin acids (Lesage and Perrault, 1993) or minimally degraded derivatives which are likely aerosolized as solids when rosin is heated to vaporization and cooled rapidly (as in soldering).

Possibly due to inadequate characterization of airborne solids derived from heated rosin, formaldehyde exposure is widely used as a surrogate when measuring personal exposure to airborne "rosin core solder pyrolysis products" (Burge, 1984; U.S. Department of Labor, 1989; Lesage and Perrault, 1993). Use of the formaldehyde standard protects against irritation, but the actual correlation between airborne solids and formaldehyde may not be significant (Burge et al., 1981; Burge, 1984; Lesage and Perrault, 1993), and remains undefined.

A review of existing and past workplace air contaminant standards for rosin-derived contaminants reflects the uncharacterized nature of

the contaminants. Existing standards are based exclusively on exposure to formaldehyde, produced as rosin is thermally degraded (usually during soldering of electrical connections and circuits). Irritation from formaldehyde is cited as the significant criterion in setting exposure standards and guidelines for exposure to airborne "rosin core solder pyrolysis products" (U.S. National Institute for Occupational Safety and Health, 1988; U.S. Department of Labor, 1989; American Conference of Governmental Industrial Hygienists, 1992). Prior to a judicial decision staying enforcement of updated Permissible Exposure Limits (PELs), the U.S. Occupational Safety and Health Administration (OSHA) PEL for "Rosin Core Solder Pyrolysis Products" was given as 0.1 mg/m^3 , measured as formaldehyde (U.S. Department of Labor, 1989). The U.S. National Institute for Occupational Safety and Health (NIOSH) Recommended Exposure Limit (REL) is 0.1 mg/m^3 , 15 min ceiling, measured as formaldehyde (U.S. National Institute for Occupational Safety and Health, 1988). No American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV) is cited in the 1994–1995 TLV booklet, although exposure reduction to levels "as low as possible" is recommended (American Conference of Governmental Industrial Hygienists, 1994). Australia, Belgium, and the United Kingdom also regulate exposures to "Rosin Core Solder Pyrolysis Products" at 0.1 mg/m^3 (as formaldehyde) (International Labour Organization, 1994). NIOSH testimony to OSHA examined the proposed PEL for rosin core solder pyrolysis products as a formaldehyde issue and recommended the ceiling limit based upon NIOSH views of formaldehyde as a carcinogen (U.S. National Institute for Occupational Safety and Health, 1988). In recognition of the need to examine exposure to airborne resin acids, the ACGIH notes: "the TLV Committee currently has this substance under review, because the decomposition products, including the resin acids (colophony), have been identified as substances that produce occupational asthma and irritation" (American Conference of Governmental Industrial Hygienists, 1992).

Due to the demonstrated role of resin acids as a class in dermal sensitization and the observed pulmonary sensitization from heated rosin, resin acids should be examined as possible pulmonary sensitizers produced during the use of rosin flux. To accomplish this, a direct sampling and analytical method is needed to quantify solids derived from heated rosin, and if possible, the actual resin acid compounds present in a worker's breathing zone.

1.1. Proposed sampling and analytical method to measure rosin aerosol

A well-defined, direct and reproducible sampling and analytical method to evaluate exposure to aerosol derived from heated colophony will enable the collection of exposure data and correlation with outcomes such as occupational asthma. There are three desirable characteristics of an acceptable analytical method for airborne resin acids and other rosin-derived solids: (1) the method must be sensitive to a potentially complex and variable assortment of airborne colophony solid contaminants; (2) the method should be reasonably simple and relatively inexpensive; and (3) an acceptable method will also allow recognition and quantification of specific resin acid compounds collected. These points form the basis for the sampling and analytical method presented in this paper.

A well-defined sampling and analytical method for coal tar pitch volatiles is the basis for proposed sampling and analytical methodology to measure airborne resin acids: OSHA Method 58 (U.S. Department of Labor, 1986) collects airborne solids derived from heated "coal tar pitch." Coal tar is obtained by the distillation of coal, and the pitch is composed of a high percentage of polynuclear aromatic hydrocarbon (PNAH) compounds (The Merck Index, 1983a). Coal tar pitch and rosin share several similar characteristics: both are soluble in non-polar solvents and are essentially insoluble in water. Resin acids, like many coal tar PNAH compounds are expected to rapidly recondense after vaporization, providing filterable particulate air contaminants. Fig. 1 provides examples of a variety of resin acids poten-

REPRESENTATIVE RESIN ACIDS AND POLYCYCLIC AROMATIC HYDROCARBONS

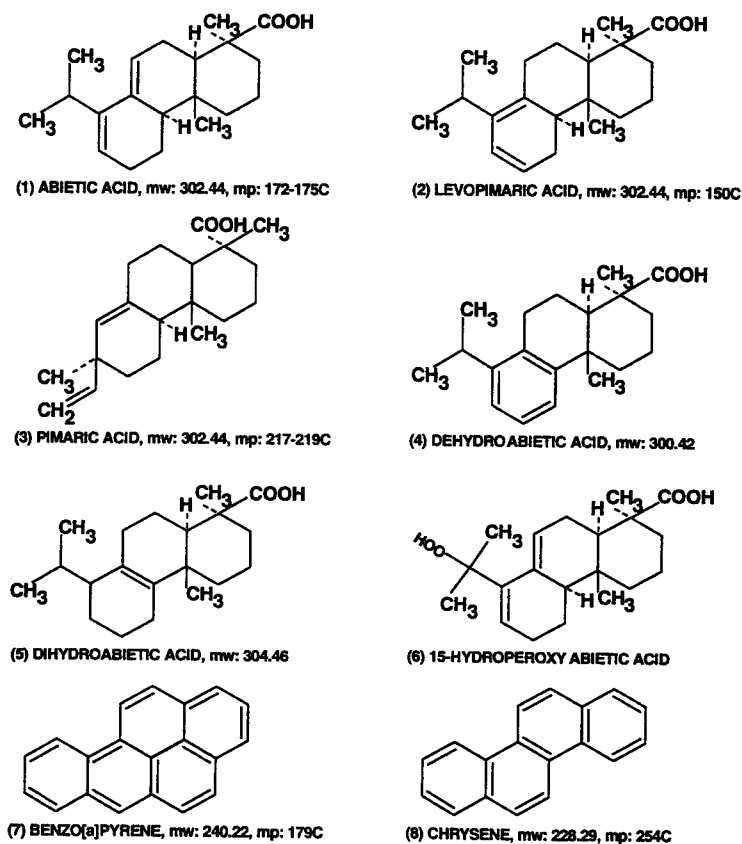


Fig. 1. Representative resin acids and polycyclic aromatic hydrocarbons.

tially found in rosin as well as several PNAH compounds.

OSHA Method 58 sampling collects airborne particulates on a glass fiber filter at a flow rate of 2.0 l/min. Analysis provides gravimetric data through benzene extraction of the filter and vacuum-heating of a portion of the extract in a tared cup to provide an estimate of "benzene soluble material" trapped on the filter. A portion of the extract is also available for further analytical work such as high performance liquid chromatography (HPLC) with an ultraviolet detector (used when analyzing for polynuclear aromatic hydrocarbons of interest in coal tar pitch vol-

atiles). The analogous approach explored in this work is to subject a portion of the filter extract to HPLC analysis using methodology already developed for analysis of the major components of unmodified rosin in technical products (Ehrin and Karlberg, 1990).

2. Materials and methods

2.1. Preliminary personal samples

Preliminary personal samples were collected in accordance with OSHA Method 58 collection media and flowrates (U.S. Department of Labor,

1986). Samples were collected during microminiature soldering and repair of laminated aircraft electronics cables and associated connectors. This work involved intermittent-to-steady soldering with the workers often using a stereomicroscope to provide low power magnification of the connections being soldered. The soldering involved rosin core solder and rosin flux (rosin dissolved in isopropyl alcohol). These operations were chosen for sampling because the authors did not have access to "assembly line" type electronics soldering, where potential maximum exposures to rosin flux by-products might be expected. These worker exposures were thought to be representative of a lesser but still significant potential due to the use of the stereomicroscopes (which placed the worker breathing zone almost directly into the visible soldering fume). Sample duration ranged from 270 to 365 min, and encompassed essentially all exposure for sampled personnel on a given day.

Measuring 37 mm in diameter with 1.0 μm pore size, binder-free glass fiber filters (SKC brand) as received from the manufacturer were used with personal sampling pumps (Dupont P2000, Gilian "Gil-Air") to collect these preliminary personal samples. Pumps were calibrated before and after sampling, and with all samples collected, the "before" flowrate was within at least 5% of the "after" flow rate. Several deviations from Method 58 were taken for the preliminary samples: (1) during collection, samples were not protected from light (a precaution to prevent photochemical changes to the polycyclic aromatic hydrocarbons normally sampled with Method 58); (2) for these personal samples only, sample and blank filters were shipped in sampling cassettes to an American Industrial Hygiene Association-accredited U.S. Navy industrial hygiene laboratory and removed by laboratory personnel prior to analysis; (3) the laboratory uses spectrophotometric grade cyclohexane for filter extraction instead of benzene, for safety purposes (Harrison and Thomas, 1987).

2.2. Analytical sensitivity, gravimetric method

Filter and solvent blanks were examined in the author's laboratory before spiked samples were

prepared to produce the analytical calibration curve for gravimetric analysis. Seemingly high filter blank levels and high filter blank deviation about the mean blank values, were observed with glass fiber filters as received from the manufacturer. Soxhlet extraction of filters removed soluble material on the filters prior to further gravimetric method precision and limit of detection determinations. Clean-up of the filters was carried out using dichloromethane (DCM) and was allowed to proceed for at least 12 h.

Several mass values (with five spiked glass fiber filter replicates each) were prepared to produce an analytical calibration curve for the gravimetric method. A known mass of Brazilian colophony was dissolved into pesticide grade DCM (Curtis Matheson Scientific) in a glass volumetric flask, bringing the total volume of the solvent/colophony mixture to a pre-determined level, yielding a 2.0 mg/ml solution. The Brazilian colophony was obtained as a representative sample of rosin used by a major U.S. manufacturer of rosin-core solder. Glass fiber filters were spiked with known volumes of this solution, allowed to dry, and were then subjected to the extraction procedures described in the following paragraph.

The analysis to estimate analytical sensitivity of gravimetric samples derived from rosin involved the following procedures: 2-ml polytetrafluoroethylene (PTFE) cups (Cahn Scientific) were cleaned using an ultrasonic bath, with tetrahydrofuran (THF), for 5-10 min and were then rinsed twice in clean pesticide grade THF (Curtis Matheson Scientific). The cups were placed onto a numbered aluminum marker, which was placed into an oven preheated to 40°C under 20" Hg vacuum for 1 h. The cups were allowed to cool to room temperature and were then weighed to the nearest microgram on a precision analytical balance (Cahn Scientific, Model C-31). The spiked glass fiber filter to be analyzed was placed into a pre-cleaned scintillation vial with a PTFE-lined cap, and 3.00 ml of pesticide grade DCM was added. The scintillation vial was then sealed with a lid and the filter/vial was shaken for 60 min. Pre-cleaning of the scintillation vials involved rinsing three times with about 3 ml of pesticide

grade THF, followed by complete drying of the vials. After extraction, each sample extract was then filtered through a 13-mm pure PTFE filter (SKC, 5 μ m pore size), held in a stainless steel holder (Cole-Parmer) attached with a Luer-Lok/reg/ fitting to a glass syringe barrel. Between analyses of filters with different spiked mass, the syringe barrel, filter holder and filter were disassembled and sonicated in THF for 1–2 min, followed by rinsing in pesticide grade THF, followed by drying of the reassembled system with ambient air under pressure. The air was introduced at about 10 psi through a hole in a rubber stopper fitted into the syringe barrel. The filter extract was then filtered through the above system to remove any non-dissolved particulate from the extract with a clean PTFE filter. This was followed by a rinse with about 3 ml pesticide grade DCM, and gentle compressed air drying between each sample analyzed. A 1.00 ml portion of the resulting filtrate was then placed into a tared PTFE cup, which was placed into the preheated oven at 40°C. The oven was taken to a vacuum of 15" Hg, with a small amount of air flow into the oven allowed for 2.0 h to clear out solvent vapors. During the last hour of oven evaporation, the air flow was secured, but oven temperature and vacuum were maintained as before. Following cooling of the cups in a desiccator for about 10 min, the cups were reweighed to the nearest microgram, and results were recorded.

The outside areas of cups and the weighing area of the microbalance were brushed with an anti-static brush prior to weighing. Also, in addition to the cups receiving filter extract, two other types of blanks were routinely run: solvent blanks, consisting of cups treated as described above, except that 1.00 ml of pesticide-grade DCM was placed directly into these cups. Balance blanks were treated as above, except that no solvent of any kind was added to these cups between weighings.

The preceding analytical procedures deviate from OSHA Method 58 in three ways: (1) dichloromethane was used as the extraction solvent instead of benzene; (2) filtered ambient air was used instead of nitrogen to push the extract

through the 13-mm PTFE filter; (3) the amount of solvent placed into the PTFE cups for evaporation was 1.00 ml instead of 1.5 ml.

2.3. Chromatographic separation and identification of collected abietic acid

The methods described by Ehrin and Karlberg (1990) were used to accomplish a separation of resin acids collected from several personal samples. The following chemicals and reagents were used in this procedure: abietic acid, technical grade (Aldrich Chemicals), acetonitrile, HPLC grade (Aldrich Chemicals), acetic acid, analytical grade (Aldrich Chemicals), and methanol, HPLC grade (Aldrich Chemicals).

An abietic acid standard was prepared from 102.50 mg of the acid dissolved in 10.00 ml of 50:50 (v/v) acetonitrile and methanol mixture. A calibration curve was prepared by diluting this solution with the same mixture to final concentrations of 0.103, 1.03 and 10.3 mg/ml. Three personal samples were collected as described above under "Preliminary Personal Samples", and sample extracts were prepared as described for the determination of sensitivity of the gravimetric method. A portion of extract was then subjected to HPLC separation. Prior to analysis, the extracts were reconstituted with 50/50% (V/V) acetonitrile and methanol mixture.

The HPLC system used was a Hewlett-Packard (HP) model 1050 fitted with a HP OD-5A column packed with 5 μ m spherical C-18 bonded silica particles and with a diode array detector set at 242 nm for abietic acid. The diode array detector was additionally set to collect the full range of 190 to 350 nm spectra during the run to assist in method development. A solution of acetonitrile (70% in volume) and a gradient mixture of 70 mM acetic acid (30 to 25% at the 10 min point) and methyl alcohol (0 to 5% at the 10 min point) was used as the mobile phase, with a flow rate of 1.5 ml/min and approximately 1500 psi of pressure at 30°C. The mobile phase ratio was held at the 10 min concentration until the end of the chromatographic run. Methyl alcohol was introduced to alleviate crystallization of resin acids which was noted during the method devel-

opment phase. An initial test run with a 10- μ l injection was found to saturate the detector at the high level standard. A 5- μ l injection of the 10.3 mg/ml solution (51.5 μ g total abietic acid) was found to give a suitable maximum response.

3. Results

3.1. Preliminary personal samples

The modifications to the OSHA sampling method described in Materials and methods (Section 2.1) were judged to be insignificant for the preliminary samples, as the major purpose of these samples was to evaluate potential for total exposure to compounds soluble in non-polar solvents during soldering. Photo-reactions could chemically alter rosin-derived solids trapped on the filter but should not significantly alter the weight of the recovered material. Sample results reported by the laboratory for the 13 samples ranged from below 33.1 μ g/filter (the reliable quantitation limit reported in OSHA Method 58) to 7.95 mg/m³. An apparently lognormal distribution of sample results was graphically confirmed using methodology of Leidel et al. (1977),

Table 1
Analytical sensitivity: gravimetric method
Mass recovered (μ g)

	Solvent blanks	Filter blanks	Pre-extracted filter blanks
	0	37	8
	0	22	10
	0	40	6
	6	61	7
	6	34	12
	3	100	16
	0	46	10
Mean	2	49	10
S	3	26	3
R.S.D.		0.53	0.34

and is shown in Fig. 2. The geometric mean for these samples was 0.352 mg/m³ with a geometric standard deviation of 5.67 mg/m³.

3.2. Analytical sensitivity, gravimetric method

As shown in Table 1, filter blanks, extracted as described in Materials and methods (Section 2.2)

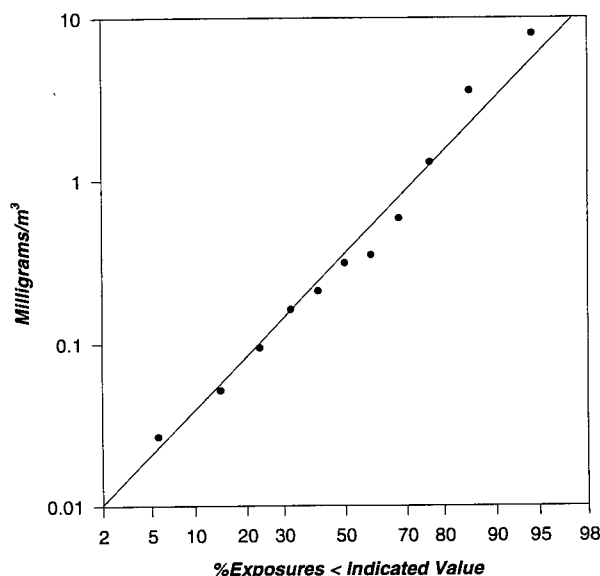


Fig. 2. Preliminary field samples, cyclohexane soluble solids. Exposures during microminiature soldering with rosin flux/rosin-core solder. Gravimetric analysis-OSHA method 58, personal samples, fully daily exposure (non-TWA), sample duration from 270 to 365 min, non pre-extracted filters used. Geometric mean = 0.352 mg/m³, geometric standard deviation = 5.67.

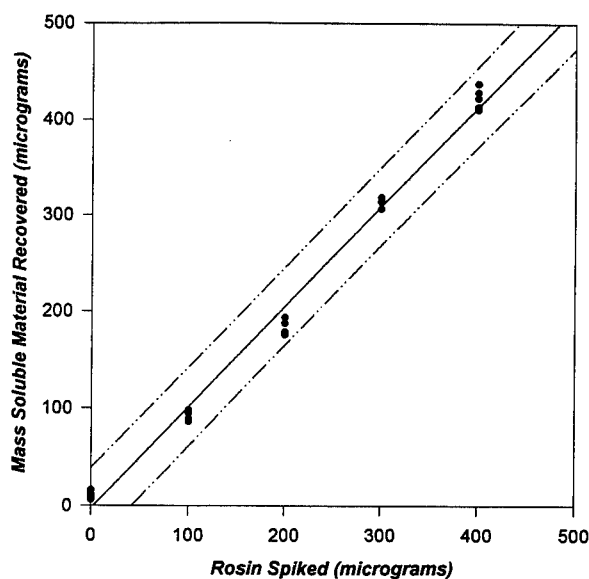


Fig. 3. Calibration curve—gravimetric analysis. $n = 5$ for each mass value spiked, for blanks $n = 7$. $y = 1.04x - 2.23$, $S_b = 3 \mu\text{g}$; $\text{LOD} = 10 \mu\text{g}/\text{filter}$ using $\text{LOD} = kS_b/\text{slope of calibration curve}$; 99% prediction intervals shown.

for determination of analytical sensitivity, showed a marked improvement in both reduction of the blank signal as well as improvements in the dispersion of filter blank replicates when pre-extracted filters were used. The mean filter blank mass recovered was reduced from 49 to 10 μg per filter. Also, a decrease of the standard deviation (S) of filter blank replicates, from 26 to 3.4 μg was observed when comparing pre-extracted filters with filters used as received. The S values of pre-extracted and non-pre-extracted filter blanks were calculated to be significantly different using a one-tailed F -test ($P < 0.025$). As the two types of filters had different S -values, a direct t -test between the replicate means for the two types of filter blanks was not possible. Using a t -table, the two-sided 99% confidence interval for the pre-extracted filter blank mean was estimated at $10 \pm 4.7 \mu\text{g}/\text{filter}$, well below the mean of 49 $\mu\text{g}/\text{filter}$ observed for the non-pre-extracted filter blanks. The reduction in filter blank variation brought about by pre-extraction gave a decrease in the relative standard deviation (R.S.D.) of the measurements from 0.53 to 0.34. In the portion of the study involving solvent blanks, where 1.00 ml

of pesticide grade DCM was added directly to seven pre-tared PTFE cups, the solvent blank mean recovered mass was 2.1 μg with $S = 2.9$.

Fig. 3 provides the analytical calibration curve produced for gravimetric analysis of rosin-spiked filters based upon five replicates at each concentration, except for blanks where $n = 7$. In one of the simpler estimates of the limit of detection (LOD) recommended by Long and Wineforder (1993), the LOD is equal to $3S_b/m$, where S_b = the blank standard deviation, and m = the slope of the analytical calibration curve. If a slope of 1 is assumed, then non-pre-extracted filters would yield a theoretical LOD of 77 $\mu\text{g}/\text{filter}$ (a calibration curve based upon non-pre-extracted filters was not prepared, due to the high S value of the non-pre-extracted filter blanks). Using pre-extracted filters with the measured reduction in S_b and the analytical calibration curve obtained, the corresponding LOD was determined to be 10 $\mu\text{g}/\text{filter}$. Based upon the preceding, the use of pre-extracted filters with their significantly improved precision may be warranted if low levels of airborne rosin-derived material are expected in the workplace to be sampled.

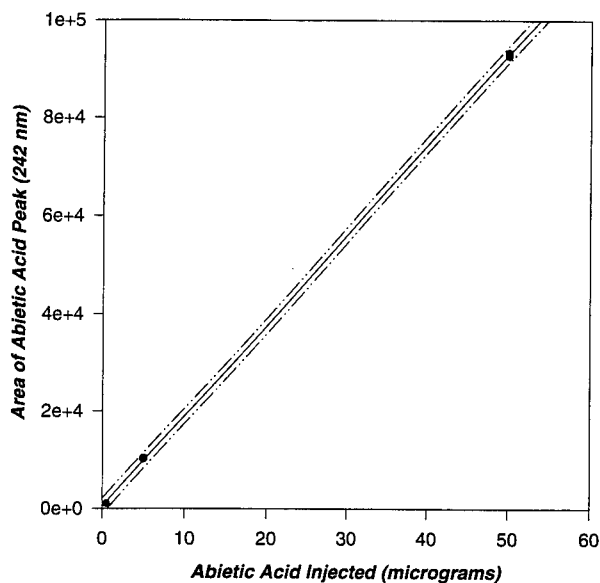


Fig. 4. Calibration curve, abietic acid—HPLC analysis. $n = 3$ for each mass value injected onto column; $y = 1848x + 503$, $S_{0.5\mu\text{g}} = 7.9$ area units; $\text{LOD} = 13$ ng using $\text{LOD} = kS_b/\text{slope of calibration curve}$; $S_{0.50\mu\text{g}}$ as S_b , $k = 3$; 99% prediction intervals shown.

Use of balance blank cups showed good agreement between replicate balance blank cups on a specific analytical oven run. Average balance blank pre- and post-differences were usually only about 1 or 2 μg (lighter) after an oven run, although on one run the post balance blank average was 26 μg lighter. This difference could be attributed to moisture driven off in the vacuum oven. We attempted to control this variable through the use of a dessicator to store cups before weighing after initial cleaning and vacuum heating. The average balance blank difference for a given oven run was added back to all cups weighed on that run. The general close agreement between the two balance blank cups in each run provided control against variables which were not quantifiable, such as balance drift and moisture.

3.3. Chromatographic separation and identification of collected abietic acid

An initial calibration run of the HPLC at the previously mentioned conditions was completed using the three standard levels. The results are found in Fig. 4.

Abietic acid was detected in at least one of three personal monitoring samples collected and analyzed. However, the concentration of the abietic acid was below the LOD of the HPLC at the given conditions. In order to detect abietic acid in the sample, a large volume injection (100 μl) was necessary. Such a large injection caused a significant shift in retention time. However, the iso-absorbance spectra were consistent with abietic acid standard.

A stable automated analytical method with improved detection parameters for abietic acid and other colophony residues is in preparation.

4. Discussion

Environmental surveillance has been cited as “the most effective means of identifying problem areas, directing control efforts, and subsequently measuring the impact of prevention strategies” (U.S. National Institute for Occupational Safety and Health, 1986). In order to accomplish this, monitoring of exposures from heated rosin should focus on compounds likely to be present in the airborne material, especially those known

to possess sensitizing properties. To date, airborne resin acid concentrations have not been examined in this context. Analytical work with compounds derived from heated rosin is summarized below: gas chromatography work by Drugov and Murav'eva (1976) characterized low molecular weight carboxylic acids and aldehydes vaporized by heating rosin with a soldering iron. Guenier et al. (1984) measured 2,4-dinitrophenylhydrazine (DNPH) adducts of acetone, acetaldehyde and formaldehyde along with other relatively low molecular weight compounds produced by thermal decomposition of rosin at several temperatures and adsorption on DNPH-coated silica gel in a pyrex sampling tube. The resulting DNPH derivatives were measured by gas and liquid chromatography. Osina (1975) used a spectrophotometric method to measure "colophony aerosol" from colophony used in high temperature fiberglass production. This non-specific method involved acetone, ethanol or acetic acid extraction of sample filters, reaction with *p*-(dimethylamino)-benzaldehyde in concentrated sulfuric acid, and spectrophotometry at 455 nm.

Burge et al. (1981) postulated that as the major fraction of rosin and known sensitizers, intact resin acids were likely responsible for pulmonary sensitization from heated rosin, as occupational asthma was found in a rosin core solder manufacturing plant where rosin was heated to temperatures of only 140°C, insufficient for pyrolytic decomposition. Reported in the same paper, Burge et al. attempted to measure the compounds thought to be directly responsible for pulmonary sensitization, rejecting formaldehyde measurements commonly used at that time (as currently). Instead, sampling and analytical procedures based upon Osina's non-specific method to measure "colophony aerosol" exposure were used. An attempt was made to correlate breathing zone aldehyde and colophony levels measured simultaneously. Colophony levels in air reportedly ranged from 0.01 mg/m³ to 3.44 mg/m³ with poor correlation between measured colophony levels and those of aldehydes. The researchers reported that higher airborne colophony levels "accompanied exposure to

heated colophony and subjective estimates of the fume." Sampling rates, volumes and specific details of "colophony" analytical methods were not specified. Airborne aldehyde levels were reportedly sampled with a 0.05% solution of 3-methyl-2-benzothiazolone hydrochloride which forms a blue cationic dye after reaction with aliphatic aldehydes in an acidic solution. Subsequent spectrophotometric analysis for aldehydes was performed using the "modified method" of Hauser and Cummin (1964).

The authors agree with Burge's assessment (1984) that formaldehyde exposure is not the desired variable to examine in issues of pulmonary sensitization from heated rosin but do not agree with the use of a non-specific measure such as the spectrophotometric measurements for airborne colophony described in the preceding paragraph. As different resin acids and other compounds potentially present may absorb energy at differing wavelengths, spectrophotometric colophony measurements will vary depending on the exact resin acids and other constituents actually aerosolized, which could be quite variable.

The bulk of the work performed regarding the identification and sensitizing potential of colophony resin acids has been in the field of contact dermatitis (skin sensitization). Hausen et al. (1989) report guinea pig skin sensitization from several "chromatographically pure" resin acid compounds (two of which are shown in Fig. 1) potentially present in colophony: abietic, podocarpic, levopimaric, and tetrahydroabietic acids were shown to be capable of sensitization. Karlberg et al. (1985), using chromatographic methods, purified commercially available abietic acid and found the chromatographically pure compound to be a greatly reduced sensitizer in the guinea pig model. It also produced no reaction among 10 human subjects sensitized to colophony (Karlberg et al., 1985). Further work by Karlberg et al. (1988) chromatographically isolated 15-hydroperoxyabietic acid from "commercial quality" Portuguese colophony and showed this reportedly stable compound to be a strong skin sensitizer in the guinea pig model. Hausen et al. (1990, 1993) examined 20 com-

pounds produced by photo-oxidation of rosin and resin acid compounds through exposure to sunlight for periods of up to 6 months. Two of these compounds were considered significant based upon the amounts recovered and their sensitizing ability: 8,12-peroxydo- Δ 13(14)-dihydroabietic acid and 12-hydroxyabietic acid.

Similar sampling and analysis methods as used in NIOSH Methods 5506 and 5515 for polycyclic aromatic hydrocarbons were considered for this work. These methods employ PTFE filters to collect PNAH particulate and XAD-2 resin tubes to capture vapor phase PNAH components, followed by extraction of the filter with one of four solvents and desorption of the XAD-2 resin tubes with toluene. The NIOSH methods offer extraction with either acetonitrile, benzene, cyclohexane, or DCM, based upon "maximized recovery" of area samples (which must be submitted along with the personal samples and extracted before analysis of the personal samples to select the best solvent for the particular material sampled). Following extraction, aliquots of the samples are analyzed by HPLC or capillary column gas chromatography (U.S. National Institute for Occupational Safety and Health, 1994a,b) Although potentially more thorough due to the use of a solvent chosen specifically for the material sampled, and the use of a sorbent tube to capture vapor phase contaminant, the NIOSH methods do not provide for total gravimetric analysis of the solvent soluble material (although this data could be provided from the NIOSH methods by additional use of OSHA Method 58 procedures after extraction). Also, the NIOSH methods are more complex as written with eight area samples required to provide data for selection of the extraction solvent. The analysis of the sorbent tube (front and back sections) with yet another solvent adds significantly to the cost and complexity of these methods. Potential sublimation (The Merck Index, 1983b) of the smaller molecular weight PNAHs (2-3 ring compounds, i.e. naphthalene, anthracene, and to a lesser extent flouranthene and phenanthrene) off the PTFE filter makes this aspect of the NIOSH method necessary if one wishes to include these

compounds in analysis. However, rosin components such as abietic and other resin acids are not expected to enter the vapor phase at room temperature, and filtration alone (as in the proposed adaptation of OSHA Method 58 for their capture and analysis) should be sufficient.

Concerning the use of DCM instead of benzene as specified by Method 58, cyclohexane has been proposed as a replacement for benzene in the gravimetric analysis of coal tar pitch volatiles due to the health concerns associated with benzene exposure (Harrison and Thomas, 1987). Cyclohexane was found by Hekmat et al. (1994) to provide significant solvation of coal tar pitch, although benzene and dichloromethane were better suited for solvent extraction of glass fiber filters spiked with coal tar pitch volatiles. dichloromethane recovered a broader range of compounds than cyclohexane and a greater mass of coal tar pitch material than either benzene or cyclohexane. Benzene was not chosen as the filter extraction solvent in this study. Benzene soluble materials must be examined for OSHA compliance questions concerning coal tar pitch volatiles (by definition of the OSHA PEL), although any other suitable solvent could be used, and others may be preferable as recognized by the NIOSH methods 5506 and 5515 previously cited. In the interest of uniformity, we chose to use dichloromethane due to its commonly acknowledged solvent properties and its non-flammability, as opposed to benzene with its attendant health and flammability risks (Aksoy et al., 1974; Vigliani, 1976), and cyclohexane, which is also quite flammable.

Future work to characterize resin acids and other rosin-derived solids collected on glass fiber filters should more closely follow OSHA Method 58 with respect to protection of sample from light and use of nitrogen instead of filtered ambient air to filter sample extracts. These steps are not critical if a compound is expected in relatively high concentrations (such as abietic acid, which is a major component of rosin), or if only gravimetric information is desired. However, complete characterization of compounds present on the filter should be carried out with these

precautions to minimize photochemical reactions and air oxidation of trapped analyte.

5. Conclusion

Our proposed sampling and analytical procedure based upon OSHA Method 58 is sensitive to solvent-soluble particulate produced during soldering with rosin flux. The presence of resin acids in a personal breathing zone sample was confirmed using an HPLC technique. The exposure distribution for total solvent-soluble material seen from the 13 personal breathing zone samples gives a glimpse at the gross exposure problem posed by the use of rosin flux. Additional work should be undertaken on a larger scale with efforts to correlate measured exposures and reported medical effects. Additional laboratory efforts are needed to more fully characterize airborne resin acids and their derivatives to which workers are exposed. This should be followed by toxicological evaluations of the compounds found. A gravimetric standard for airborne resin acids may be desirable if gravimetric exposure data can be correlated with adverse health effects. The gravimetric standard may be made contingent upon the recovery and chromatographic determination of a level of abietic or other resin acid deemed significant. This would be similar to the current OSHA standard for coal tar pitch volatiles, which depends upon the chromatographic analysis of one or several PNAH compounds at levels in excess of the PEL (U.S. Department of Labor, 1989).

Acknowledgements

The opinions and assertion contained herein are those of the authors and are not to be construed official or reflecting the views of the Navy Department or the Naval Service at large, nor of other government agencies. The authors wish to thank the following individuals and organizations for involvement in this project: U.S. Army Medical Research Detachment at Wright-Patterson Air Force Base; Mr. William Dinigui and Mr. Eugene Suit of Naval Hospital, Jacksonville, Florida; Mrs. L.R. Connolly and HM2

Patricia Valenti, USN. The authors would also like to thank Ms. Lana Martin for the technical editing of this manuscript. This work was supported by Naval Medical Research Institute Detachment (Toxicology) and Department of the Navy Research Task NEHC 1323.

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ELSEVIER

Toxicology 111 (1996) 239-249

TOXICOLOGY

Exposure-response functions in Air Force toxic risk modeling

Lloyd L. Philipson*, James M. Hudson, Alex M. See

ACTA Incorporated, 23430 Hawthorne Blvd., Skypark no. 3, Suite 300, Torrance, CA 90505, USA

Abstract

A new methodology for estimating the probabilistic risk from acute toxic exposures is planned as a support tool for the Air Force at the Eastern and Western Ranges. Two such methodologies are programs entitled the Launch Area Toxic Analysis program (LATRA) and the Cold Spill Toxic Risk Analysis program (COSTRA). These programs combine probabilistic models of an accident (when applicable), release cloud formation and dispersion (appropriate to the toxic substance and accounting for meteorological conditions), and new exposure-response functions (ERFs) for sensitive and normal exposed populations. These ERFs, anchored on specific exposure standards, estimate the probability of a given severity of health effect in a particular population as a function of the concentration or dose to which it is exposed. The further development and acceptance of these ERFs by the toxicology community, especially for different sensitivities, are key concerns addressed in this paper.

Keywords: Exposure-response functions (ERFs); Toxic risk; Rocket launches

1. Introduction

Methods for estimating the probabilistic risk from acute toxic exposures are planned to provide new support capabilities for the Air Force at the Eastern and Western Ranges. LATRA is the Launch Area Toxic Risk Analysis program. COSTRA is the Cold Spill Toxic Risk Analysis program. LATRA develops the mean number of casualties and the complete risk profile (curve of the probability of exceeding each possible number of casualties) from the toxic effluents in normal and failed missile and space launches.

COSTRA similarly treats toxic materials storage and handling accidents.

The programs are intended to assist the Air Force in ensuring health and safety while avoiding undue limitations of deterministic exposure standards. This will be done by replacing worst-case modeling of exposed areas ("toxic corridors") with predictions of the probabilities of possible numbers of affected individuals. Exposures, especially just above a standard, that could impose a launch hold, on a risk basis may merely imply small probabilities that small numbers of individuals could be affected to a minor degree. Furthermore, the programs produce risk characterizations in the same terms as for other launch

*Corresponding author.

hazards, allowing for commonality in risk-based launch decisions.

LATRA and COSTRA combine probabilistic models of an accident (when applicable), release cloud formation and dispersion (appropriate to the toxic substance and accounting for meteorological conditions), and new exposure-response functions (ERFs) for normal and sensitive exposed populations. These ERFs, anchored on specific exposure standards, estimate the probability of a given severity of health effect in a particular population as a function of the concentration or dose to which it is exposed. The further development and acceptance of these ERFs by the toxicology community, especially for different sensitivities, are key concerns focused upon in this paper.

2. LATRA

The LATRA program has been developed for the Air Force to provide comprehensive assess-

ments of casualty risks from possible toxic emissions from a space or missile launch (Haber et al. 1992; Hudson et al. 1993; Chrostowski et al. 1994; Conley et al. 1994). As illustrated in Fig. 1, LATRA's procedures are:

- (1) Prompt the user to enter or modify the input data via menu options. The data files include the vehicle failure rates file, the rawinsonde meteorological profile, and the population library containing population counts or percentages by *receptor* (population center) area, sensitivity category, and shelter type.
- (2) Initiate a *scenario* with the occurrence of a normal launch, with toxic exhaust (hydrogen chloride, HCl) from solid booster rockets, if present; or of a particular accident mode, involving a conflagration or deflagration and the formation of a toxic cloud of HCl and nitrogen tetroxide (N_2O_4) (disassociating to nitrogen dioxide, NO_2), occurring at a particular altitude in flight, with the mode's corresponding probability.
- (3) Randomly sample relevant meteorological

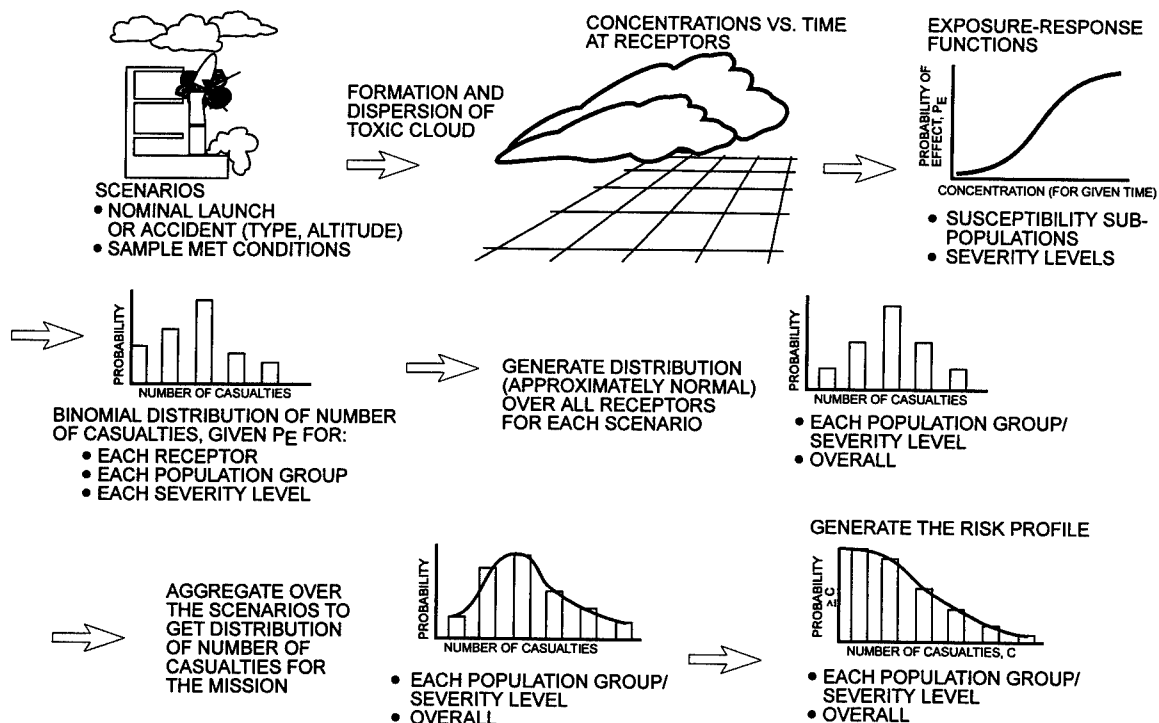


Fig. 1. LATRA toxic risk modeling.

parameters' values to continue each scenario with a specific sample case of these values, accounting for the correlations in them.

(4) Run a dispersion model (such as REEDM) (Bjorlund, 1985) with the normal launch or accident and meteorological parameters selected as inputs, to predict for each given emission the resulting distribution of concentration versus time of the toxic substance of concern (HCl and/or NO_2) across the set of exposed receptors.

Model the concentrations that result in the interiors of several types of structures at the receptors.

(5) At each receptor and for each structure type, apply an *exposure-response* model to each population subgroup or subpopulation (at present: children, aged, and bronchitics; healthy adults) with a particular level of susceptibility to health effects from the toxic substance, and for each considered level of severity of such health effects (at present: at least mild, at least significant, fatal).

Derive thereby the probability of occurrence of each level of severity of effect for each subgroup, versus their predicted level of exposure (peak or time weighted average concentration, or dose, as appropriate). (If exposure to a combination of several materials is involved, derive the combined probability of effect). Fig. 2 exhibits examples of exposure-response functions that have been developed thus far. At each receptor, this probability is the parameter of a binomial distribution for the total number of individuals in the subgroup at the receptor who suffer at least that severity of effect.

(6) Convolve the binomials by adding their means and variances at the receptors to obtain the mean and variance of an overall approximating normal distribution (based on the Central Limit Theorem) of the total number of the people suffering some severity of effect. (Alternative means for obtaining the convolutions can be applied when the population sizes and exposures are such that the normal approximation is not satisfactory.) A distribution has now been derived for the number of casualties for each subgroup/severity and for the total population for the scenario that began with a particular accident

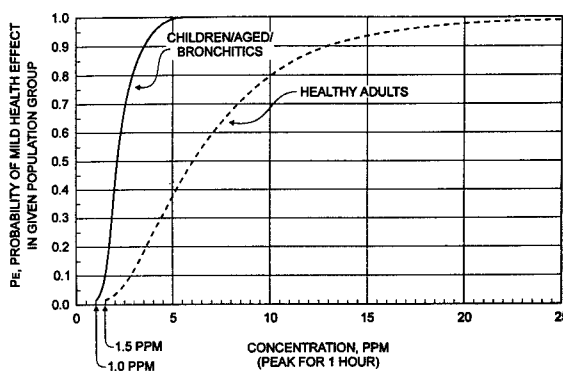


Fig. 2. Examples of log-probit exposure-response functions for NO_2 .

mode, if applicable, vehicle altitude, and case of meteorological parameter values.

(7) Aggregate these distributions over the scenarios by discretizing them and then adding their frequencies in each discrete interval weighted by the probabilities of occurrence of the scenarios. The results are aggregated discrete distributions for the mission. The weighted sum of the means of the scenarios, i.e. the mean of each mission distribution, is the mission *casualty expectation*, E_C , for each subgroup/severity and overall.

(8) Derive a *risk profile* for the mission for each subgroup/severity and for the overall population from the corresponding distributions by computing for each given number of casualties the sum of the probabilities of all numbers of casualties equal to or greater than the given number.

(9) Express the effects of multiple toxic exposures from a given launch accident in combined risk profiles by developing joint probabilities of effect from the individual toxics' probabilities of effect (assuming their independence). This assumption is probably conservative because of the high level of correlation that can be expected among the individual toxic exposures. However, it neglects any possible synergistic effects of the multiple exposures.

(10) Apply the joint probabilities of effect to the population categories.

(11) Generate the resulting casualty distributions and risk profiles as before.

The foregoing procedures have been implemented and risk profiles produced for a number of launches at the Eastern and Western Ranges. Examples are shown in Figs. 4 and 6. These and all subsequent risk profiles should be considered illustrative only, with the object of illuminating the difference to health and safety management between deterministic hazard and probabilistic risk criteria. The profiles exhibit the probability per launch of any possible number of children, aged, and bronchitics, or of healthy adults, suffering at least a given severity of effect from one-hour exposures to HCl at concentrations with predicted peaks. The expected numbers of people in each subpopulation and overall populations who suffer an effect (i.e. at least mild) are the E_C values shown.

The examples shown are for a Peacekeeper ICBM launch at Vandenberg Air Force Base.

Fig. 4 shows the profiles for a normal launch of the large solid fuel rocket. Fig. 6 shows the profiles for a modeled accidental conflagration on the pad. (Only HCl is considered in this example). Figs. 3 and 5 show the corresponding deterministic isopleths, computed with the REEDM dispersion model (Bjorklund, 1985) for several levels of peak concentration. (In operational applications, in order to account for wind direction uncertainty, sectors $\pm 20^\circ$ about the isopleths are defined as "toxic corridors" to be avoided when their exposure levels exceed specified standards). Comparing the set of isopleths with the corresponding sets of risk profiles evidences how the risk modeling, especially when specifications of the size of the sensitive subpopulation and of potential sheltering are taken into account, can ease the launch decisions.

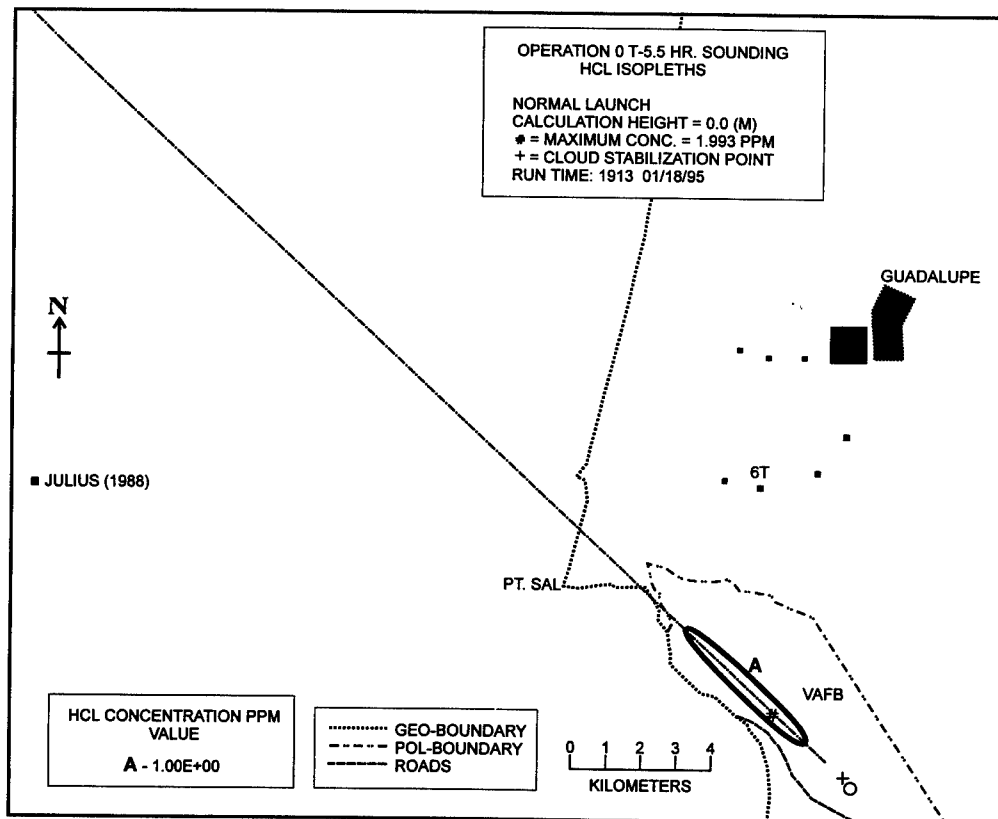


Fig. 3. Predicted HCl isopleths for a normal launch of Peacekeeper, Vandenberg AFB, 1-18-95, T-5.5 Hr. weather forecast.

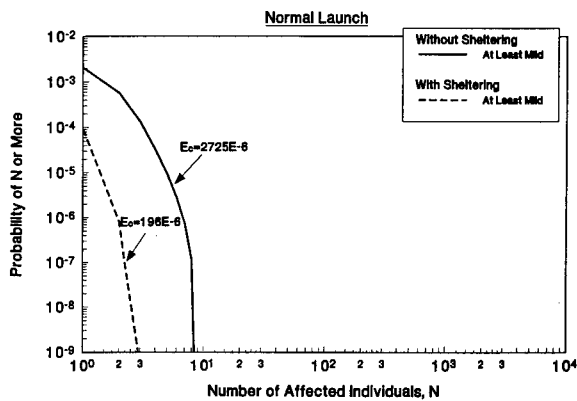


Fig. 4. Risk profiles for the effect of HCl for normal launch of Peacekeeper, Vandenberg AFB, 1-18-95, T-5.5 Hr. weather forecast.

3. Exposure-response functions

The ERFs comprise the key module of LATRA or COSTRA. An ERF translates dispersion model output for individual or combined airborne toxic substances into the probability of a given health effect on an individual at a given location, with a given susceptibility, to a given level of severity. The specification of ERFs and assessments of the uncertainties in them is the central topic of this paper. The properties and multiple factors of ERFs are elaborated upon in greater detail below.

3.1. Properties of ERFs

Fig. 7 shows examples of alternative ERFs that have been employed to the present. They define,

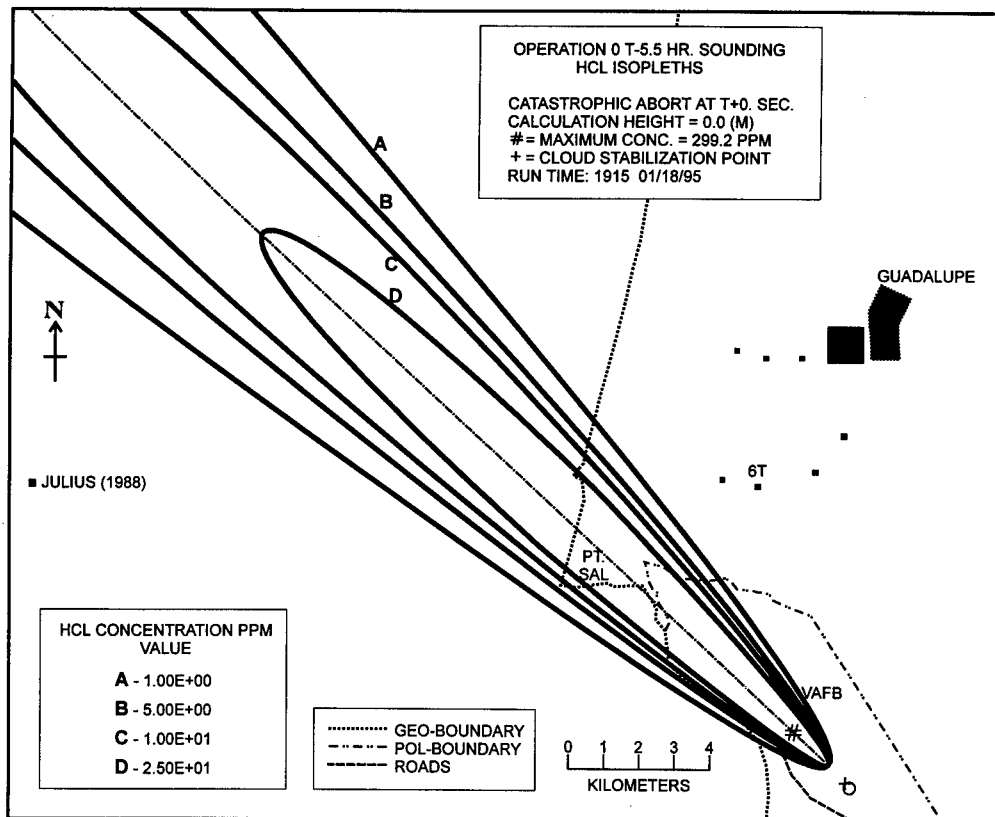


Fig. 5. Predicted HCl isopleths for an aborted launch (conflagration of pad) of Peacekeeper, VAFB, 1-18-95, T-5.5 Hr. weather forecast.

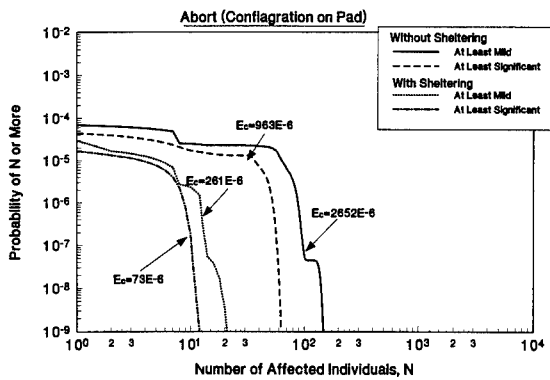
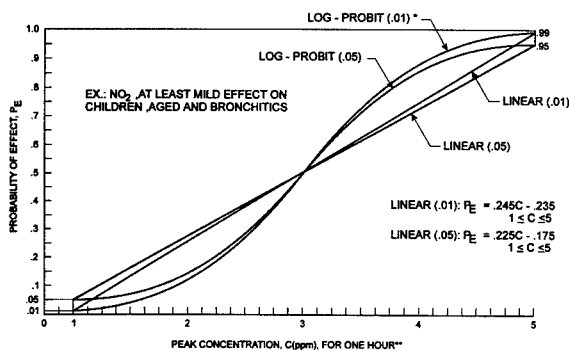


Fig. 6. Risk profiles for the effect of HCl for Peacekeeper aborted launch 1-18-95, T-5.5 Hr. weather forecast.

for exposures over a one-hour period to any given concentration of nitrogen dioxide (NO₂), the probability that an individual in one of the two susceptibility subgroups or subpopulations that have been treated will suffer *at least* a mild health effect. Working definitions of the subpopulations and effect severity levels are discussed later.

Log-probit (equivalent to log-normal) functions of the character described in Eisenberg et al. (1975) and ten Berge et al. (1986), for example, are one generic form for ERFs. Estimates of symmetric lower and upper percentiles specify them. The fundamental procedure for estimating these lower and upper percentiles is employing all available information to make defensible judg-



* LOG-PROBIT IS EQUIVALENT MATHEMATICALLY TO LOG-NORMAL FUNCTION SPECIFIED BY ESTIMATES OF INDICATED PERCENTILES

** GENERALLY, DIFFERENT ERFs REQUIRED FOR DIFFERENT TIMES OF EXPOSURE

Fig. 7. Examples of alternative exposure-response functions.

ments of: (1) the exposure level *below which essentially no one in the subpopulation would suffer the given severity of effect*; and (2) the level *above which essentially everyone would*. Such judgments may be considered *integrations* of all of the various kinds of judgments now made in the modeling of toxic responses to establish exposure standards (National Research Council, 1982). In Fig. 7, the lower percentile estimate for the sensitive subpopulation is the NRC Committee on Toxicology's (COT's) Short-term Public Emergency Guidance Level (SPEGL), 1 ppm ceiling over one hour of exposure to NO₂.

Extended linear functions that pass through the origin are alternative ERF forms that require the estimation of only one percentile. Note that they are conservatively above the log-probit (and essentially any other applicable form) in the low exposure range.

The functions are cut off at the percentiles, becoming 0.0 below the lower percentile and 1.0 above the upper percentile, in response to the assumption that the two percentiles are specified, respectively such that essentially no one is affected at lower concentrations and essentially everyone is affected at higher concentrations.

Finally, depending on the toxic substance, Time-Weighted Average Concentration, or integrated dose, may replace Peak Concentration as the independent variable. Also, if exposure time variation is important, this is taken into account by defining a set of ERFs for each of a selection of exposure times.

For carcinogens, linear functions without threshold cut-offs that pass through the 0.01 percentile and the origin have been employed. The 1/10000 level is based on the guidance level for "acceptable" life-time incremental risk of cancer from a single exposure (Committee on Toxicology, 1984-88).

3.2. ERF factors

The factors that enter into the specification of ERFs are:

- Analytical form of the function
- Selection of the independent variable, with its pertinent exposure time
- Definition of the susceptibility subpopulations

- Definition of the health effect severity levels
- Estimation of the function's required percentiles (or other parameters)
- Analysis of uncertainties

The resolution of these factors is accomplished in the present methodology by expert judgments tempered by relevant data availability. These judgments, the processes by which they were made, and the capacity for their improvement are the central concerns of this article.

3.2.1. Analytical form

It has been seen that log-probit and linear functions have been treated up to now. The log-probit has precedent in some previous toxic response modeling (Eisenberg, 1975; ten Berge, 1986; Davies, 1989). It is particularly easy to quantify if two symmetric percentiles are estimated. The linear function can be a useful conservative approximation when an estimate of only a single percentile, such as a maximum exposure standard, is available. This form is the preferred model for carcinogens. Other forms are possible, including the logistic and Weibull. The most suitable form depends in general on the toxic substance and the characteristics of the exposed population. It is feasible with LATRA and COSTRA to include assessments of the variations in their outputs as they may result from the use of different possible forms. It is likely, however, that only unimportant variations will result at low exposure levels.

3.2.2. Independent variable

The ERFs defined thus far for HCl and NO₂ exposures of the general public have been anchored on the COT's SPEGL, interpreted as a ceiling concentration over one hour of exposure. Time-weighted average concentrations have been proposed by (USAF) Armstrong Laboratory for application in the Three-Tier Model (Table 1) (Poitras, 1993) for certain hydrazines in "cold-spill" accidents, and for other than the more sensitive individuals. Dose (integrated concentration over time that may include an amplification exponent on the concentration) is another alternative for the independent variable of an ERF.

The choice depends, of course, on a judgment of the nature of the physiological response to the toxic substance (von Niedling, 1979; von Niedling and Wagner, 1979; Clewell et al., 1988). Different responses for different exposure times may require a set of ERFs for each of a selection of times over the range of concern.

3.2.3. Susceptibility subpopulations

At present, two subpopulations have been discriminated as to their sensitivity to exposure to HCl and N₂O₄/NO₂: (1) children, aged, and bronchitics; and (2) healthy adults. Reasonable judgments were able to be made of their ERFs' lower and upper percentiles on the basis of the experimental data and their available interpretations (Clayton and Clayton, 1982; Committee on Toxicology, 1984–88; Guzelian et al., 1992). Equally important census and epidemiological

Table 1
Recommended limits for use in the three-tier model^a

N ₂ O ₄ (as NO ₂) ^b
Tier 1: 25 ppm (1/2 IDHL), ceiling
Tier 2: 5 ppm (EPA LOC), ceiling
Tier 3: 1 ppm (SPEGL), ceiling
UDMH (also applies to Aerozine-50)
Tier 1: 25 ppm (1/2 IDHL), ceiling
Tier 2: 5 ppm (EPA LOC), ceiling
Tier 3: None
MMH (also applies to Aerozine-50)
Tier 1: 25 ppm (1/2 IDHL), ceiling
Tier 2: 0.52 ppm (SPEGL), 1 h TWA
Tier 3: None
Hydrazine
Tier 1: 40 ppm (1/2 IDHL), ceiling
Tier 2: 2 ppm (SPEGL), 1 h TWA
Tier 3: None
Hydrogen chloride ^b
Tier 1: 50 ppm (1/2 IDHL), ceiling
Tier 2: 5 ppm (TLV), ceiling
Tier 3: 1 ppm (SPEGL), ceiling

^aPopulations (on-base) exposed to Tier 1 levels or above warrant immediate isolation/evacuation; those exposed to Tier 2 levels or above have some risk of adverse effects to average individuals; those exposed to Tier 3 levels or above have some risk of adverse effects to sensitive individuals.

^b"Hot spill" substances treated by LATRA. Others "cold spill" hypergols treated by COSTRA.

(Evans et al., 1987) data were available that enabled estimates of the numbers of individuals in these two subpopulations in the areas neighboring the launch facilities. These estimates were perhaps of the same order of accuracy as the population estimates made for the analyses of the other launch hazards.

The issue of present concern, and the main issue of this conference, is the potential for improvement in the delineation of the subpopulation as a function of the toxic substance. It has been proposed, for example that differences in susceptibility can be expected depending on gender and ethnicity. Other categorizations may be possible and worthwhile to evaluate. However, unless, (1) the associated ERF percentile judgments can be made for each of the categories, and (2) the census and other data required to estimate the sizes of such subpopulations are available, the finer categories would have to be reaggregated for the risk analyses. The loss of information that would result could possibly be compensated for by extending the ranges of uncertainty considered for the ERF percentiles.

3.2.4. Health effect severity levels

Three levels have been defined thus far for HCl and N₂O₄/NO₂ exposures:

- Mild: no organic damage, temporary irritation
- Significant: organic damage, treatment required
- Fatal: irrelevant for possible exposures in launches

These definitions are evidently ambiguous to a degree and should be revisited and refined if necessary. Nevertheless, they have reasonable relationships to the definitions of a casualty for the other launch hazards. Most importantly, they have loose but meaningful associations with interpretations of the available experiment data and judgments about them reported by the COT and others (e.g. Clayton and Clayton, 1982; National Research Council, 1994).

As with the delineation of susceptibility subpopulations, increasing the resolution of the severity levels is worthwhile providing that the ERF percentiles can be estimated meaningfully for each level.

3.2.5. ERF percentile estimates

The appendix to this article contains summaries of the derivations of the percentile estimates made for the HCl and NO₂ log-probit ERFs assumed to date. These, as well as the other ERF factors that have been discussed, are under review and may be expected to be refined.

The present HCl and NO₂ percentile estimates are reasonable judgments of the peak concentration over a one-hour period of exposure such that, as has been said, on the one hand essentially no one in a given susceptibility subpopulation would suffer a given level of severity of health effect and, on the other hand that essentially everyone would. The judgments are anchored on the COT's SPEGL as the lower percentile for a Mild effect or worse in sensitive individuals (children, aged, bronchitics). They otherwise derive from the animal and human experimental data and interpretations published in the reports on the COT's considerations of HCl and N₂O₄/NO₂ (Committee on Toxicology, 1984-88), supplemented by references in Patty (in Clayton and Clayton, 1992), and checked against data in IRIS (Calabrese and Kenyon, 1991). Other studies have recently been made of the effects of HCl and their probabilities, which may add to the data (Patrick, 1991; Kamrin, 1992). The results to date (Haber et al., 1992; Hudson et al., 1993; Conley et al., 1994) are summarized in Table 2.

For other toxic substances, including the hydrazines, only linear ERFs have been defined thus far. The single percentile estimates they require for each subpopulation and severity level have been established either as SPEGLs where they are available, or in relation to the EPA's LOCs and NIOSH's IDLHs (US EPA, 1987; Clewell et al., 1988; Chrostowski et al., 1994).

ERFs remain to be considered for the combined effect of multiple toxics. In the present risk modeling, the individual ERFs have been retained. A compound probability of effect is then derived under the assumption that the individual effects operate independently. This is conservative in the derivation of the number of individuals affected to a given severity level. However, it neglects the possible synergy of the effects that may increase the level of severity above the

Table 2
Summary of present estimates of percentiles of NO₂ and HCl ERFs*

Subpopulation	NO ₂				HCl			
	Mild		Significant		Mild		Significant	
	1%	99%	1%	99%	1%	99%	1%	99%
Children, aged, bronchitics	1	5	3.5	67	1	3	3	30
Healthy adults	1.5	25	5	100	3	30	30	300

*Peak concentration, ppm; 1-h exposure.

individual levels. Further consideration of these factors is anticipated.

3.2.6. Uncertainties

No risk analysis is complete without an assessment of the effect of data and, as feasible, modeling uncertainties on the outputs. LATRA and COSTRA provide effective vehicles for quantitative uncertainty analyses. In particular, the uncertainties in the judgments made to specify a set of ERFs can be evaluated as to their impacts on the values of casualty expectation and the risk profiles produced by the models. The implication to the acceptability of the unavoidably imprecise ERF judgments is important. The range of opinions of the toxicologists who induce the judgments can be treated explicitly as a range of uncertainty. In a formalized procedure involving a panel of experts, if this proves to be desirable, the range can be reviewed and iteratively revised. The final result will be bounds on the LATRA/COSTRA predictions that will illuminate the confidence with which a launch decision can be made.

4. Conclusion

LATRA and COSTRA are intended to provide significant new risk management tools for the two ranges. In particular, LATRA can provide a new dimension of flexibility for the Range Commanders in their launch commit decisions by replacing deterministic overly-conservative hazard limits by probabilistic assessments of potential casualties. A critical need for the application

of these models, however, is the establishment of exposure response functions that are recognized and accepted by the toxicology community. This article has proposed how this may be done effectively.

Acknowledgements

The authors wish to express their appreciation for the support provided to them in the work described in this paper by the Safety Directorates, 30th and 45th Space Wings, Vandenberg Air Force Base and Cape Canaveral Air Force Station, and by Jon Collins and Jerold Haber of ACTA, Inc. who provided valuable reviews of the methods and their results.

The investigations described in this paper were performed under the sponsorship of the U.S. Air Force 30th and 45th Space Wings, Safety Directorates, Contract No. FO4703-91-C-0112, but their results are neither approved nor disapproved by the Air Force.

Appendix: Estimates of ERF percentiles for NO₂ and HCl NO₂ estimates (one-hour exposure) COT data

- Fatal C₅₀, 174 ppm (Referencing Book, 1983).
- Mild reversible to irreversible pulmonary function effects, up to 5 ppm.
- Short-term or substantial chronic tissue damage, functional impairment, aggravation of other disease processes, 5 to 100 ppm.
- Irreversible lung damage or death, above 150 ppm.

(e) Increase in airway resistance (healthy and bronchitic), reversible if single exposure, about 1.5 ppm.

(f) Decreased diffusing capacity and increase in alveolar-arterial pO_2 (oxygen partial pressure) difference, reversible if single exposure, about 5 ppm.

(g) Recommended Short-term Public Emergency Guidance Level (SPEGL), 1 ppm.

Patty data (Clayton and Clayton (1982), referencing the American Council of Governmental and Industrial Hygienists (ACGIH) and the World Health Organization (WHO))

(h) Fatal pulmonary edema, 100 ppm (ACGIH)

(i) Pulmonary edema with lung lesions, 50 ppm (ACGIH)

(j) Respiratory irritation and chest pain, 25 ppm (ACGIH)

(k) Increased airway resistance, 3-6 ppm (WHO)

Percentile estimates for healthy adults

Fatal: C_{50} , 174 ppm (data item (a) above); C_{01} , 100 ppm (h)

Intermediate probability, 150 ppm (d)

Statement (d) is considered consistent with statements (a) and (g), but does not add new information for the percentiles.

Note that C_{50} is given not C_{99} . However, from a property of the lognormal,

$$C_{99} = C_{50}^2 / C_{01} = 300 \text{ ppm}$$

Significant: C_{01} , 5 ppm (c); C_{99} , 100 ppm (c)

Intermediate probability, 50 ppm (i)

As above, (i) is considered consistent with (c).

Mild: C_{01} , 1.5 ppm (e); C_{99} , 25 ppm (j)

Intermediate probabilities, 5 ppm (b), (f) and 3-6 ppm (k)

Percentile estimates for children, aged, and bronchitics

Item (e) indicates a 1.5 ppm airway-resistance-increased threshold for bronchitics (as well as healthy) individuals. The COT has established a SPEGL of 1 ppm that presumably safeguards the sensitive subpopulation, bronchitics and children and the aged, still more. A conservative view is adopted here to replace for the sensitive subpopulation the healthy adults' 1.5 ppm C_{01} with the SPEGL of 1 ppm (g), a one-third decrease in value. This same decrease is applied to the per-

centiles of the exposure-response function for significant or fatal effect on healthy adult individuals to obtain reasonable, probably conservative, corresponding percentiles for bronchitics and children. For the 99-percentile for mild effect, 5 ppm is chosen based on items (b) and (c). The results are:

Fatal: C_{01} , 67 ppm; C_{99} , 200 ppm

Significant: C_{01} , 3.5 ppm; C_{99} , 67 ppm

Mild: C_{01} , 1 ppm; C_{99} , 5 ppm

HCl estimates (one-hour exposure)

COT data

(a) Short-term Public Emergency Guidance Level (SPEGL) = 1 ppm.

(b) Mouse RD_{50} (median dose giving 50% decrease in respiratory rate) ≈ 309 ppm. This (rounded to 300 ppm) corresponds to intolerable sensory irritation and incapacitation in humans.

(c) 0.1 Mouse $RD_{50} \approx 30$ ppm causes slight stinging and burning of eyes, nose, and throat.

(d) 0.01 Mouse $RD_{50} \approx 3$ ppm causes very slight to no irritation.

(e) The COT recommends a maximum concentration of 20 ppm for one hour for a worker Emergency Exposure Guidance Level (EEGL).

(f) The COT recommends 100 ppm for a 10 min EEGL.

(g) 1000-2000 ppm is "dangerous for even short exposures" to humans.

Patty data

(h) Human volunteers found concentrations of 50-100 ppm for 1 h barely tolerable (consistent with (c)-(b) range above)

(i) Throat irritation occurred at 35 ppm for brief exposures (consistent with (e) and (c) above).

(j) 10 ppm was considered maximum concentration for long exposures (not inconsistent with (d) above).

(k) Immediate irritation of nose and throat, but no lasting effects, occurred at 5 ppm (consistent with (d) above).

Percentile estimates for healthy adults

Mild: C_{01} , 3 ppm (d); C_{99} , 30 ppm (c)

Significant: C_{01} , 30 ppm (c); C_{99} , 300 ppm (b)

Fatal: C_{01} , 1000 ppm (g)**; C_{99} , 2000 ppm (g)**

Percentile estimates for children, aged, bronchitics

Mild: C₀₁, 1 ppm (a); C₉₉, 3 ppm (d)

Significant: C₀₁, 3 ppm (d); C₉₉, 30 ppm (c)

Fatal: C₀₁, 300 ppm*; C₉₉, 600 ppm*

Notes: *Obtained from "healthy adults" values by dividing by 3, the ratio of the "healthy adults mild effect" 1 percentile to the COT's SPEGL that protects the most sensitive subpopulation.

**Probably conservative, since these concentrations are considered "dangerous," not necessarily fatal.

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SESSION V
INCORPORATING SUSCEPTIBILITY
INTO RISK ASSESSMENT



ELSEVIER

Toxicology 111 (1996) 253–270

TOXICOLOGY

Obtaining information about susceptibility from the epidemiological literature¹

Jean A. Grassman

School of Public Health, Columbia University, New York, NY, USA

Abstract

Whether people become ill after encountering environmental pollutants depends on the magnitude of their exposure and their capacity to respond. Exposure and intrinsic response capabilities vary within the population. Those that become ill when the general population remains largely unaffected are considered to be highly susceptible. The U.S. Environmental Protection Agency (USEPA), responsible for protecting the public from environmental pollutants, has developed risk assessment procedures to assist in evaluating the likelihood of health effects. However, the Agency's ability to evaluate the risk faced by highly susceptible populations is often hindered by the paucity of adequate health effects data. Response variability can be assessed with animal models and human epidemiological studies. Although animal models are useful when evaluating the effect of gender and developmental stage on susceptibility, inbred rodent strains underestimate the genetic and lifestyle-induced variability in susceptibility found in human populations. Epidemiological approaches are the preferred source of information on variability. This paper reviews the epidemiological literature from the perspective of a risk assessor seeking data suitable for estimating the risk to highly susceptible populations. Epidemiological approaches do not measure the full range of population response variability. Rather, "susceptibility factors" are evaluated either as risk factors or by focusing on the susceptible population, e.g. children. Susceptibility factors due to genetics, developmental stage, gender, ethnicity, disease state and lifestyle are most frequently encountered. Often, the information describing the health impact of the susceptibility factor is incomplete due to, (1) a failure to consider factors modifying susceptibility; (2) inadequate exposure data; (3) a failure to evaluate the health impact of the susceptibility factor. In addition, for a given exposure agent, several susceptibility factors may be relevant. While incomplete data describing susceptibility factors limits the opportunity for quantitative estimations of risk, available information can supplement qualitative evaluations and risk management.

Keywords: Risk assessment; Variability; Susceptibility; Dose response; Risk factors

*Corresponding author, National Institute of Environmental Health Sciences, Laboratory of Biochemical Risk Analysis, P.O. 12233 Mailcode C4-05, Research Triangle Park, NC 27709, USA. Tel.: 919 941 3466; Fax: 919 541 3647; E-mail: grassman@niehs.nih.gov.

¹Paper submitted for the Conference on Risk Assessment Issues for Sensitive Human Populations, 25–27 April 1995, Wright-Patterson Air Force Base.

1. Introduction

The U.S. Environmental Protection Agency relies on risk assessments to predict the likelihood of adverse health outcomes and make decisions about permissible levels of contaminants. Yet, pollutant-induced illness continues to affect highly susceptible members of the population. Permissible levels of respirable air particulate increase the incidence of asthma-related hospital visits (Schwartz et al., 1993) and the prevalence of bronchitis in asthmatics (Dockery et al., 1989). Evidence that subpopulations continue to become ill after compliance has been attained has led to a reevaluation of the scientific and procedural underpinnings of the risk assessment process.

At their most fundamental level, risk assessments use existing toxicological *response* data as a standard curve to estimate health effects at projected levels of *exposure*. A complete estimate of the distribution of risks requires that variability in toxicological response as well as exposure variability be incorporated into the risk assessment.

Variability in exposure has been acknowledged in risk assessments by incorporating point estimates representing different locations in the projected exposure distribution. Risk estimates may be based on the maximally exposed individual (MEI) (USEPA, 1992), the reasonable maximum exposure (RME) which is the magnitude of exposure that should not be exceeded by more than 5-10% of the population (USEPA, 1989) and the theoretical upper bounding estimate (TUBE) which is the level of exposure expected to exceed all individual exposures (USEPA, 1992). An alternative to point estimates is to use the exposure variability to model probability distributions of risk (Bogen and Spear, 1987; Lambert et al., 1994).

Response variability is difficult to measure in human populations and until recently, risk assessment guidelines have not provided detailed procedures for estimating the risk for susceptible populations. Cancer risk assessment guidelines assume that all members of the population have equivalent responses (USEPA, 1986). Risk assess-

ments for non-carcinogens account for response variability by dividing by an uncertainty factor (Barnes and Dourson, 1988). These policies are undergoing re-evaluation and current directives acknowledge the contribution of response variability to risk and promote the evaluation of exposure and risk to susceptible individuals and populations (USEPA, 1995a,b). Despite the historical lack of guidance for incorporating information on susceptible populations into risk assessments, risk assessors have generally used data from the most sensitive population when it is available. The failure to estimate risk to susceptible populations is due to more to deficiencies in the toxicological database than to inadequate risk assessment policies.

Data on response variability can be obtained from either animal models or human epidemiological studies. Animal models are often the only source of dose response information and so have been invaluable in risk assessments. Due to extensive inbreeding, commonly used strains of mice are more than 97% genetically homozygous (Phelan et al., 1992) which limits their suitability as models of human response variability. Factors suspected of modifying risk, particularly those with a genetic basis such as cytochrome P450 polymorphisms, often have no exact rodent equivalents (Gonzalez and Gelboin, 1993).

Due to conflicting requirements, the study designs used with animal models cannot easily be modified to evaluate variability. Carcinogenesis bioassays require statistically significant cancer rates which is accomplished with dose ranges that approach the maximum tolerated dose (Lai et al., 1994). Nongenetic contributors to variability, such as stress, disease, and diet are kept to minimum and are rarely studied as independent variables.

While many of the disadvantages of response data from animal models can be avoided by using human data, there are also disadvantages to epidemiological approaches. The observational rather than experimental nature of epidemiology results in investigations that are subject to the vagaries of access to records, timing, exposure monitoring records, and subject recall.

This paper describes the susceptibility data obtained from epidemiological studies by summarizing, (1) how variability is described and the relationship between *population variability and susceptibility factors*; (2) the *categories of susceptibility factors* that contribute to differences in response; and (3) whether *epidemiological studies provide the dose-response information needed to calculate the risk to susceptible populations?*

2. Population variability and susceptibility factors

Phenotypic heterogeneity in human populations is readily apparent. Height, weight, skin, hair and eye color are highly variable due to both genetic and environmental factors. Biological response to contaminants is also heterogeneous because of differences in uptake, absorption, distribution, biotransformation and excretion capabilities. Variability also depends on the groups being compared. Breathing rates, which determine uptake, vary 1.8 to 2.5-fold between adults (Ashford et al., 1990) and 65-fold between adults and infants (Kacew, 1992). Biotransformation by detoxifying enzymes is also highly variable. Interindividual differences in enzyme activity exceed 1000-fold for cytochrome P450 2D6 which metabolizes tobacco smoke nitrosoamines (Kaisary et al., 1987) and 350-fold for glutathione-S-transferase- μ which metabolizes arene oxides (Bell et al., 1992). When the variability in each of the steps leading from exposure to health outcome is combined, large differences in susceptibility may be possible (Hattis and Silver, 1994).

How do epidemiological studies measure susceptibility and variability? In order to understand the nature of the data presented in epidemiological studies, it is instructive to construct the idealized cumulative incidence curve as shown in Fig. 1a. In this hypothetical scenario, the level of exposure that caused illness is known for every member of a population of 500. Response variability, represented by the arrow, is the range of exposures eliciting illness. In this example, the response variability spans slightly more than 1000 exposure-years.

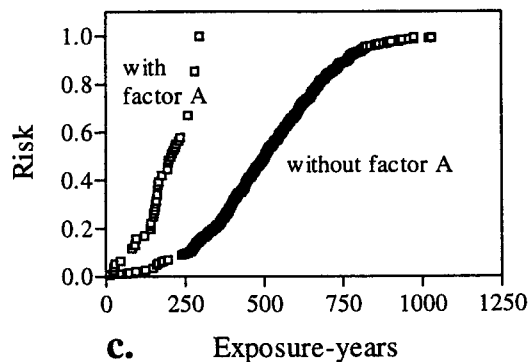
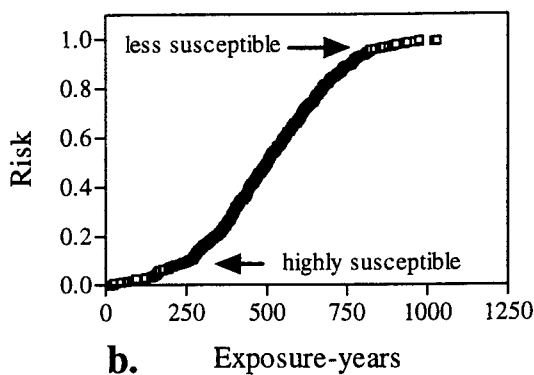
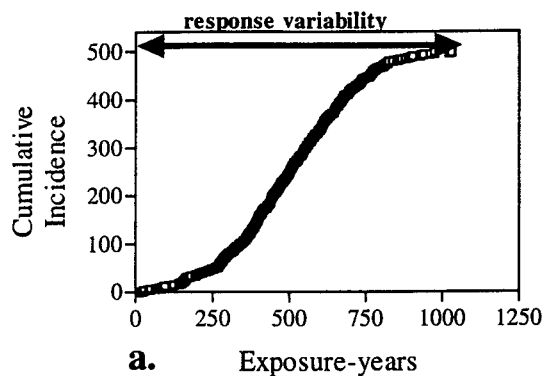


Fig. 1. Susceptibility, variability and susceptibility factors demonstrated in a hypothetical cumulative incidence curve. (a) Population response variability. (b) Susceptibility as a function of population risk. (c) Comparison of risk in populations with and without susceptibility factor A.

Fig. 1b shows the risk curve obtained by dividing the number of cases by the size of the population (Bogen and Spear, 1987). While susceptibility can be defined simply "as the state of being readily acted upon" (C. Kimmel, personal communication), it is often expressed as risk. The risk curve, then, can be used to characterize the relative levels of susceptibility present in the population. When exposure reaches 200 exposure-years, the population risk of illness is less than 0.1. Highly susceptible individuals become ill when the overall population risk is low.

While the curve in Fig. 1b appears to be ideal for estimating risk in a similar population, many aspects of susceptibility cannot be assessed. First, when considering those that became ill when the population risk was low, it is not possible to distinguish inherent susceptibility from the stochastic processes which play a role in many illnesses such as cancer (Pitot, 1993). Second, it is impossible to predict whether an individual or group is highly susceptible. While it is possible to ascertain that 10% of the population will become ill, there is no way to determine which 10% of the population is at risk. This reduces the opportunity for risk-reducing interventions.

Real-world limitations in measuring exposure adds an additional layer of uncertainty to the example. Unless exposure levels are known with a high degree of confidence, it is difficult to distinguish the relative contribution of exposure and susceptibility to illness. While a very rare disease suggests that susceptibility is a factor, overlooked exposures encountered in the workplace or through hobbies may be responsible for the illness.

In practice, the full range of variability or susceptibility indicated by the arrows in Fig. 1a and b, respectively, is a difficult quantity to measure. The upper boundary of exposure causing illness is rarely attained, while the lower boundary of exposure causing illness is difficult to observe due to the small number of people affected.

An alternative to measuring the range of variability in population response is to select subpopulations on the basis of identifiable traits and compare their risk, as shown in Fig. 1c. Compari-

son of subpopulations on the basis of identifiable factors replicates epidemiological approaches for analyzing risk factors. Out of the hypothetical population, 36 individuals who became ill when the population risk was less than 0.15 were randomly designated as having Factor A which was "suspected" of modifying the risk of illness. The remainder of the population does not possess Factor A. Comparison of the risk sustained by the two populations suggests that those with Factor A tend to be at a higher risk of illness.

Factor A is analogous to a risk factor, a variable that modifies the likelihood of disease. Risk factors measure the effect of exposure, location, and behavior as well as susceptibility. If a risk factor describes intrinsic traits related to susceptibility, the risk factor can be referred to as a "susceptibility factor".

Epidemiological approaches which measure part of the response variability as susceptibility factors have several inherent limitations. Due to the limits of sensitivity and power, only susceptibility factors having a relatively large impact on disease risk are detectable. A known susceptibility factor influences rather than determines risk. As seen in Fig. 1c, individuals without Factor A may become ill at lower exposure levels than those with Factor A.

Similarly, patients with bladder cancer from arylamine exposure include both slow (more susceptible) and fast (less susceptible) acetylators, and many exposed slow acetylators never develop cancer (Vineis and Ronco, 1992). Individual susceptibility is determined by a complex interplay of intrinsic and environmental factors. Epidemiological studies are able to identify the impact of only a few of them.

3. Categories of susceptibility factors

If risks are to be fully assessed for diverse segments of the population, all relevant susceptibility factors should be included. Most existing reviews of factors influencing susceptibility have focused on single factors such as genetics (Gonzalez and Idle, 1994), ethnicity (Kalow et al., 1986), age (Calabrese, 1986), gender (Calabrese, 1985) or susceptibility to a single category of

Table 1
Categories of susceptibility factors

Class	Susceptibility factors	Exposure	Health effect
Constitutive susceptibility factors			
Genetic	Slow acetylation	Arylamines ^a	Bladder cancer
Ethnicity	European	Sunlight ^b	Melanoma
Gender	Male	DBCP ^c	Sterility
Age	12-16 Weeks gestation	Methylmercury ^d	Neurological retardation
Acquired susceptibility factors			
Disease state	Asthma	Sulfur dioxide ^e	Bronchoconstriction
Quality of life	Cigarette smoking	Asbestos ^f	Lung cancer

DBCP, dibromochloropropane.

^aKadlubar et al., 1992.

^bReintgen et al., 1982.

^cWhorton et al., 1979.

^dMortensen, 1992.

^eJaeger et al., 1979.

^fCoultas and Samet, 1992.

health effects such as pulmonary disease (Brain et al., 1988) or cancer (Idle et al., 1992).

The categories of susceptibility factors shown in Table 1 can be classified as either constitutive or acquired. Constitutive categories of susceptibility factors, such as *genetics*, *ethnicity*, *developmental stage*, and *gender* are demographic characteristics of a population that are fixed or change in a predictable fashion, as with *developmental stage*. In contrast, susceptibility factors due to *disease state* and *quality of life* are acquired through the situations and events encountered during the course of life. The prevalence of acquired susceptibility factors may change over time as diets improve or as people take up cigarette smoking. The distinction between acquired and constitutive factors is made because the options for risk management differ. With constitutive susceptibilities, the proportion of the population with the factor is fixed. As an example, susceptibility to methylmercury due to gestational age will be a characteristic of all fetuses and so exposure must be controlled. In contrast, steps to reduce susceptibility factors that are acquired may be feasible and even desirable. For example, better understanding of the etiology and management of asthma may reduce

its role as a factor mediating susceptibility to the health effects of air contaminants such as sulfur dioxide. Similarly, cigarette smokers are highly susceptible to cancer when exposed to asbestos (Coultas and Samet, 1992). While lowering exposure is certainly desirable, reducing the size of the susceptible population by providing incentives to stop smoking is also productive.

The categories of susceptibility factors shown in Table 1 were compiled from the epidemiological literature. In addition to the well-known susceptibility factors associated with *genetics* and *developmental stage*, susceptibility factors attributable to *ethnicity*, *gender*, *disease state*, and *quality of life* were also frequently encountered. Susceptibility factors can influence the magnitude of response (e.g. level of enzyme activity) as well as the type of response possible (e.g. teratogenic effects, death of spermatogonia). An effort was made to formulate categories according to natural divisions so a given susceptibility factor falls clearly into one of the categories with a minimum of overlap. However, any individual or group will have traits that place them into more than one category (e.g. elderly white female).

Genetic susceptibility factors include genetic syndromes (e.g. ataxia telangiectasia), heritable

defects in DNA repair and tumor suppressor genes, and polymorphisms of strategic proteins. The latter includes enzymes that metabolize environmental contaminants, enzymes responsible for normal metabolic processes and mixed histocompatibility factors. Molecular biology techniques have made it possible to easily identify gene variants that modify xenobiotic metabolism. Among them is the NAT2 gene which governs acetylation and influences susceptibility to bladder cancer (Kadlubar et al., 1992).

Ethnicity often overlaps with *genetics* since gene frequencies are closely tied to ethnic origin. For example, one of the alleles conferring a slow acetylator phenotype is found only in African-American populations (Bell et al., 1993a). Non-Mendelian traits with the potential to influence susceptibility have also been identified, e.g. differences in pulmonary function (McDonnell and Seal, 1991). One of the few examples of susceptibility due to ethnicity is seen amongst Europeans who are at higher risk of developing melanoma when exposed to sunlight than Africans (Reintgen et al., 1982). In general, risk factors associated with ethnicity are prone to confounding by unidentified susceptibility and exposure factors associated with socioeconomic status.

Susceptibility factors related to reproduction were not included as an independent category but were divided into *developmental stage* and *gender*.

Developmental stage includes all stages of the life span from conception to death. As such, the category includes susceptibility factors related to prenatal development, infancy, childhood, mature adults and the elderly.

Susceptibility factors due to *gender* include but are not limited to those related to the reproductive organs and capabilities. Men exposed to dibromochloropropane (DBCP) may lose primary spermatogonia and become sterile (Whorton et al., 1979). Gender-related hormonal differences can affect susceptibility. Menopause in women mobilizes bone lead stores (Silbergeld et al., 1989) while animal models suggest that the greater susceptibility of females to dioxin-induced liver cancers is due to estrogens (Huff et al., 1994).

Disease state includes both infectious and noninfectious conditions that modify response to environmental contaminants. Previous infection with hepatitis B increases the likelihood of developing liver cancer when exposed to aflatoxin (Wu-Williams et al., 1992).

Susceptibility factors related to *quality of life* are diverse and include modifiers of host response such as stress, exercise, nutrition, exposure to other contaminants and habits such as smoking and illicit drug use.

4. Do epidemiological studies provide the dose-response information needed to calculate the risk to susceptible populations?

As summarized in Table 1, the epidemiological literature describes many examples where response to contaminants is reported to be modified by susceptibility factors. The question risk assessors face is whether the literature provides data that can be used to estimate toxicological response, preferably in the form of dose response or cumulative incidence curves. If so, a risk estimate can be formulated using the responsiveness of the highly susceptible population.

Fig. 2 shows the types of data needed to describe the toxicological response of a highly susceptible population: susceptibility factor(s), exposure, and health effects. This information can be assembled from cross-sectional, case-control or cohort studies. In practice, causal inference is difficult to establish in cross-sectional studies and most information on susceptibility factors comes from case-control or cohort studies. Case reports can be used if exposure levels were also evaluated. A well-documented case report of infant methemoglobinemia formed the basis for the EPA's reference dose for nitrate in drinking water (USEPA, 1991b).

In cohort studies, which more closely approximate experimental approaches than other epidemiological designs, the study population is chosen on the basis of exposure. As shown in Fig. 2a, factors modifying susceptibility are analyzed as risk factors. The response observed in groups with and without the susceptibility factors are

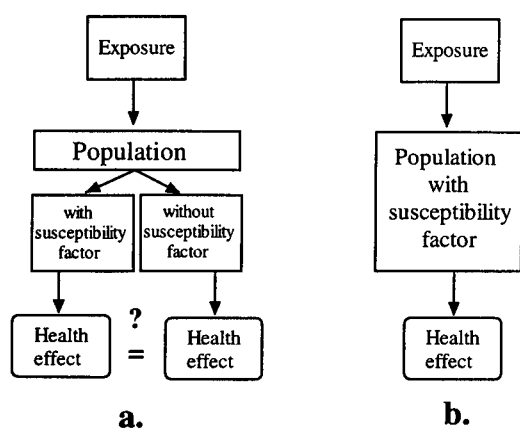


Fig. 2. Data needed to characterize the dose-response relationships associated with susceptibility factors. (a) Comparison of response in groups with and without susceptibility factors. (b) Dose-response assessed in a population possessing a factor associated with susceptibility.

usually compared as odds ratios or relative risks. Alternatively, dose response data may be taken directly from the population with the susceptibility factor, as shown in Fig. 2b. This is necessary when the susceptibility factor has no analog in the general population, as with developmental stage. Fetuses, but not adults, are at risk of developmental neurological damage when exposed to methylmercury (Mortensen, 1992).

Controlled-exposure experiments, which are a variant of the cohort design, can be used to study the short term acute effects of non-carcinogens. This design permits the selection of a study population on the basis of susceptibility factors.

In case-control studies, cases are chosen on the basis of illness and a comparable population without the illness serves as controls. The frequency or magnitude of factors associated with susceptibility can be compared in the cases and controls. The inference that a highly inducible form of cytochrome P450 1A1 modifies susceptibility to lung cancer is based on its higher frequency in patients than in controls (Nakachi et al., 1991).

Several types of the deficiencies are encountered when seeking dose-response information for highly susceptible populations. They include:

4.1. Failure to evaluate potential susceptibility factors

Epidemiological studies may fail to incorporate potential susceptibility factors into their analysis. For example, occupational epidemiological studies were reviewed for data that could be used to determine whether non-whites were at a greater risk for occupational cancers. The authors found 182 articles that included race in their analysis. Of these, 61 were case-control studies that matched on race, making it impossible to determine the effect (Kipen et al., 1991).

Controlled-exposure experiments can be used to study the acute and reversible health effects of non-carcinogens. Participants can be selected in a manner that permits evaluation of a variety of susceptibility factors. Many of the problems associated with epidemiological studies can be avoided, particularly uncertainties regarding the magnitude and duration of exposure. Since exposures are known, responses due to differences in susceptibility can be distinguished.

Ozone, one of the seven criteria air pollutants regulated by the USEPA (USEPA, 1986), is a pulmonary irritant that has been extensively studied in controlled-exposure experiments. Ozone is formed by photochemical processes and is a major contributor to the poor air quality encountered in urban areas. Despite evidence that the current allowable level may not be protective, more than 90 million Americans live in areas that *exceed* the regulatory standard (USEPA, 1993). Sensitive populations include people with asthma and children (Balmes, 1993). Pulmonary damage consistent with exposure to ozone was found in the lungs of 80% of otherwise healthy Los Angeles area youths (ages 14 to 25) who were autopsied after accidental deaths (Sherwin, 1991).

Table 2 summarizes possible susceptibility factors reported or analyzed in 32 controlled exposure studies performed between 1988 and 1994. Subjects were exposed for up to 1 h to levels of ozone near the regulatory limit of 0.12 ppm. Pulmonary function tests, measurements of airway responsiveness and indicators of inflammation in the upper and/or lower respiratory tracts were used to assess the effects of ozone exposure.

Table 2
Susceptibility factors assessed in 32 controlled studies of acute pulmonary changes detected after ozone exposure 1988-1995

Susceptibility factor	Reported		Analyzed	
	No./32	%	No./32	%
Gender				
Females only	2	(6.2)	—	
Males only	16	(50.0)	—	
Males/females	14	(40.6)	3	(9.4)
Not reported	0	(0)	—	
Age				
< 18	2	(6.2)	—	
18-39	23	(19.4)	1	(3.1)
> 40	0	(0)	—	
More than 1 age group	7	(21.9)	1	(3.1)
Not reported	0	(0)	—	
Ethnicity				
African Am/white	1	(3.1)	1	(3.1)
White	3	(9.4)	—	
Not reported	27	(84.4)	—	
Disease				
Ozone responsive	2	(6.2)	2	(6.2)
Asthma	8	(25.0)	3	(9.4)

While constitutive factors such as gender and developmental stage (age) were reported, their impact on susceptibility was usually lost since few studies included them as variables in their analyses. Over half of the studies examined males between the ages of 18 and 39 whose ethnicity was not reported.

Of the studies that analyzed potential susceptibility factors, age had the largest effect and was inversely correlated with pulmonary function (Drechsler-Parks et al., 1989; McDonnell et al., 1993). The effects of gender were contradictory. Ozone produced no differences in pulmonary function when males and female subjects were compared (Drechsler-Parks et al., 1989). In contrast, a greater decrement was found in female subjects when compared to similar studies evaluating males (Messineo and Adams, 1990). Race was evaluated in only one study and had no impact on the outcome (Seal et al., 1993) although significant differences in pulmonary baseline values have been reported between Americans of African vs. European ancestry

(McDonnell and Seal, 1991). A small proportion of the population was found to be "ozone responsive" based on the magnitude of their pulmonary deficits after exposure (Schelegle et al., 1989). Several studies examined whether asthma modified sensitivity to ozone and found no effect (Koenig et al., 1990; Weymer et al., 1994). This survey indicates that the opportunities for evaluating susceptibility factors for ozone were not fully exploited despite the use of controlled exposure study designs.

In summary, dose-response information may be unavailable because data on the risk factors modifying susceptibility were not collected or analyzed.

4.2. Incorrect identification of susceptibility factors: confounding

Risk factors can be incorrectly classified as susceptibility factors because of confounding. Confounding occurs when a variable is related both to the putative susceptibility factor and to the adverse health outcome. Ethnicity is often misinterpreted as a susceptibility factor. A notorious example is the excess lung cancer mortality detected amongst African-American coke oven workers in the years 1952-62 (Lloyd et al., 1970). The steel industry concluded that African-American workers were more susceptible to lung cancer than white workers (Draper, 1991). Subsequent analysis indicated that differences in exposures due to racially based job assignments had confounded the outcome. Of those employed at the site, 19% of African-American employees compared to 3% of white employees worked topside of the coke oven where the concentration of carcinogens was the highest (Lloyd, 1971). Similar errors are possible when considering morbidity and mortality from contemporary environmental exposures. Correlation of Toxic Release Inventory and census data suggests that African-Americans, Asians and Hispanics are more highly exposed to air contaminants than whites (Perlin et al., 1995). Future investigations of the relationship between ethnicity and air pollution will need to account for the differential exposures to avoid confounding.

The use of aggregate risk factors may also confound the relationship between a putative susceptibility factor and health effect. Socioeconomic status, annual income and level of education are aggregate risk factors which act as surrogates for specific measurements of variables related to susceptibility, exposure and lifestyle.

The role of ethnicity as a susceptibility factor for cancers with an environmental component is often obscured by the use of aggregate variables. A review of 10 studies found that the difference in cancer incidence between African-Americans and whites was nominal after adjustment with aggregate indicators based on census tract, income, level of education and urban residence (Gorey and Vena, 1994). In contrast, another study found that the differences in cancer mortality between affluent African-American and white populations persisted after adjustment by median family income, another aggregate variable. In fact, mortality rates in affluent African-Americans from Suffolk county (New York) did not differ from those found in African-Americans nationwide (Polednak, 1990).

The evaluation of ethnicity as a susceptibility factor for bladder cancer induced by cigarette smoke also produces contradictory results. The outcomes of three recent case control studies suggested that, compared to whites, African-Americans are more (Burns and Swanson, 1991), less (Harris et al., 1990) or equally (Hartge et al., 1993) susceptible to cigarette smoke-induced bladder cancer. The role of ethnicity as a susceptibility factor was evaluated by adjusting with aggregate risk factors such as marital status, professional and education (Harris et al., 1990) or occupational category (Hartge et al., 1993). The Burns and Swanson study, which found African-Americans to be more susceptible to smoking-induced bladder cancer, calculated an odds ratios based on the aggregate variable of occupational category. Neither carcinogen exposure nor susceptibility factors that modify the metabolism of bladder carcinogens were evaluated in any of the three studies.

These examples suggest that "hidden" confounders encountered when using aggregate risk factors may obscure the role of susceptibility and

exposure risk factors. As a result the role of ethnicity as a susceptibility factor for cancers in general or for specific cancers such as bladder carcinomas cannot be determined.

Confounding of susceptibility factors is not restricted to those associated with ethnicity. *Aflatoxin* and *hepatitis B* modify cancer risk by functioning as both exposure and susceptibility factors. Aflatoxin-contaminated food and hepatitis B infection each can increase the risk of hepatocellular carcinoma (HCC). Together, their effect is interactive (Wu-Williams et al., 1992). People positive for hepatitis B antigen and exposed to aflatoxin face a much higher risk of HCC than people exposed to only one of the factors. A recent hypothesis suggests that a dietary factor which increases the activation of aflatoxin, rather than aflatoxin *per se*, is responsible for the augmented cancer risk (Campbell, 1994). If so, the relationship between the susceptibility factor (hepatitis B) and health effect (HCC) may be confounded by an unidentified nutritional susceptibility factor which modifies the effect of the exposure to the carcinogen aflatoxin. If this proves to be the case, susceptibility information intended for risk assessments should include populations where both hepatitis B status and the nutritional susceptibility factor have been surveyed.

4.3. Susceptibility factors with questionable environmental etiology

The role of probable susceptibility factors in contaminant-induced asthma is particularly convoluted. Air pollutants, particularly ozone (Balmes, 1993) and environmental tobacco smoke (Chilmonczyk et al., 1993) may play role in the etiology of asthma. At issue is whether African-American children are more susceptible to asthma when exposed to air pollutants than white children. Compared to white children, asthma prevalence is 26% greater (White et al., 1994) and mortality 3-4 times higher (Marder et al., 1992) in African-American children. During periods of high ozone concentrations (exceeding 0.11 ppm), asthmatic African-American children visit hospital emergency rooms more frequently (White et al., 1994). Several alternative relation-

ships may explain these differences in the occurrence and severity of asthma:

- Susceptibility due to ethnicity: African-American children may be more susceptible to asthma than white children. If this alternative is true, environmental exposures would not play a role in the occurrence of asthma.
- Ethnic susceptibility factor: African-American children may be more susceptible to asthma after exposure to environmental pollutants than white children.
- Confounding due to other susceptibility factors: the susceptibility observed in African-American children may not be due to ethnicity *per se* but to other factors which modify susceptibility. Possible modifiers include respiratory disease due to overcrowding (Marder et al., 1992), diet, medical care, and stress (Rumbak et al., 1993).
- Confounding due to differential exposures: African-Americans live predominantly in urban areas with higher levels of ozone (USEPA, 1990). Exposure can also come from unexpected local sources. Children from low-income families without air conditioners may have higher exposures because their windows are left open during episodes of high ozone levels (White et al., 1994).
- Asthma morbidity and mortality are due neither to exposure or susceptibility factors, but to differences in disease management and access to emergency medical care.

There is currently no consensus over the role of African-American ethnicity as a susceptibility factor for the development of air pollution-induced asthma. While arguments regarding the plausibility of these alternatives can be made, the purpose of this example is to demonstrate the complexity of identifying valid susceptibility factors.

4.4. Incomplete exposure data

Epidemiological studies may document the health impact of susceptibility factors but not the associated exposures. Many xenobiotic detoxifying enzymes are polymorphic. The level of enzyme activity expressed, or phenotype, can have a great impact on metabolic capabilities. Populations with the "susceptible" phenotype of several polymorphic enzymes are at a higher risk

of lung, colon, bladder cancer than those with the "resistant" phenotypes as seen in Table 3. Activating enzymes that occur polymorphically include cytochrome P450 1A1 (CYP450 1A1), CYP450 1A2, and cytochrome P450 2D6 (CYP450 2D6) while conjugating enzymes include *N*-acetyltransferase (NAT2) and glutathione-*S*-transferase- μ (GSTM1) (Idle et al., 1992).

Although the genetic basis of enzyme polymorphisms and their impact on susceptibility have been thoroughly studied, less is known about etiologic agents. Since many cancers are relatively rare, most polymorphic genes are evaluated in case-control studies where the relevant exposures are difficult to identify because they occurred years earlier. Exceptions include cigarette smoke and CYP 1A1 (Nakachi et al., 1991), arylamines and NAT2 (Cartwright et al., 1982; Hein, 1988), and asbestos and CYP 2D6. The association between asbestos and CYP 2D6 has not been replicated in subsequent studies (Caporaso et al., 1989).

The identity of the missing exposure agent cannot be deduced. While few exposure agents have been linked to the enzymes responsible for the modified risk, *in vitro* studies indicate that many different chemicals may be substrates. Nearly 20 substrates have been identified for CYP 2D6 alone (Wrighton and Stevens, 1992). Establishing that a chemical acts as a substrate for a polymorphic enzyme does not implicate it as the unidentified exposure agent.

Although a population with specific enzyme polymorphism may clearly be at enhanced risk, the inability to establish the identity of the causal agent precludes the use of this information in risk assessments. By necessity, risk assessments, as well as risk management and the regulatory apparatus are organized around characterizing and controlling contaminants whose identity is known.

Documentation of the magnitude of exposure may be missing. Genetically conferred defects in reducing enzymes and hemoglobin variants are often cited as increasing susceptibility to illnesses caused by oxidizing agents. Populations with sickle cell trait, glucose-6-phosphate dehydrogenase (G6PD) deficiency, α_1 -antitrypsin,

Table 3
Correspondence between substrates and documented exposures in probable genetic susceptibility factors predisposing to cancer

Polymorphic gene (susceptible phenotype)	Enzyme substrates	Epidemiologically linked exposures	Cancer risk
CYP450 1A1 (highly inducible)	PAHs	Cigarette smoke	7.3 Lung ^a
CYP450 1A2 (highly inducible)	Nitrosoamines, aflatoxin, arylamines, PAHCs	Cigarette smoke	>2 Colorectal ^b
CYP450 2D6 (extensive metabolizer)	Nitrosoamines	Cigarette smoke, asbestos	2.8-18 Lung ^c
N-Acetyltransferase (slow acetylator)	Arylamines	Arylamines	2 Bladder ^d , 2 colorectal ^e
Glutathione-S-transferase- μ (deficiency)	PAHs, aflatoxins	Cigarette smoke	1.7-3 Lung ^f , 1.7-6 bladder ^g

CYP, cytochrome; PAHs, polycyclic aromatic hydrocarbons; PAHCs, polyaromatic heterocyclics.

^aCosma et al., 1993.

^bNakachi et al., 1991.

^cCaporaso et al., 1989.

^dCartwright et al., 1982.

^eHett et al., 1987.

^fNazar-Stewart et al., 1993.

^gBell et al., 1993b.

methemoglobin reductase, and beta thalassemia have been assumed to be highly susceptible despite the lack of supporting evidence (Calabrese, 1984; Ashford et al., 1990). Although erythrocytes taken from G6PD deficient individuals are extremely sensitive to oxidizing agents in vitro (Amoruso et al., 1986), the exposure levels responsible for health effects have not been reported. The USEPA shelved a recent attempt to develop a reference dose for naphthalene because only a case report lacking quantitative exposure data was available (H. Choudary, personal communication).

4.5. Incomplete documentation of adverse health outcomes

Often, putative susceptibility factors are identified before their impact on risk is investigated epidemiologically. Many susceptibility factors relating to the "quality of life" fall into this category. For instance, trace metals such as selenium, copper, and iron act as enzyme cofactors and are essential to the function of xenobiotic detoxifying enzymes such as cytochrome P450s (Sipes and Gandolfi, 1986). While it is plausible that dietary deficiencies in micronu-

trients alter enzyme function, their effect on human susceptibility has not been estimated.

The existence of factors modifying susceptibility may also be inferred from inter-individual differences in response called biomarkers. The changes detected as biomarkers may be early predictors of adverse health effects. Table 4 lists several potential susceptibility factors. The biomarkers associated with the susceptibility factors listed in the table provide evidence that there are substantial functional differences in metabolism within the population. Biomarkers measured in vitro show that approximately 11% of the population is sensitive to clastogens based on the induction of sister chromatid exchanges (SCEs) (Wiencke et al., 1991); the capacity to repair damaged guanine varies from 3- to 5-fold (D'Ambrosio et al., 1984); and erythrocytes from people with sickle cell trait and G6PD deficiency are extremely sensitive to oxidizing agents (Amoruso et al., 1986; Kuypers et al., 1990). The degree to which these differences affect risk cannot be directly evaluated because the relationship between the biomarker and adverse health effect has not been established.

Polymorphic detoxifying enzymes that have not been evaluated epidemiologically include

Table 4
Putative susceptibility factors based on differences in biomarker response

Putative susceptibility factor	In vitro treatment	Biomarker
Unknown ^a	Diepoxybutane	SCE inducibility
DNA repair capability ^b	Nitrosoamines	<i>O</i> ⁶ -Methylguanine-transferase variability
Sickle cell trait ^c	Peroxides	Erythrocyte fragility
G6PD deficiency ^d	Ozone, nitrogen dioxide	Erythrocyte glutathione depletion
Paraoxonase polymorphism ^e	Organophosphates	Enzyme variability
Sulphotransferase polymorphism ^f	Arylhydroxylamines, phenols	Enzyme variability

G6PD, glucose-6-phosphate dehydrogenase; SCE, sister chromatid exchange.

^aWiencke et al., 1991.

^bD'Ambrosio et al., 1984.

^cKuypers et al., 1990.

^dAmuroso et al., 1986.

^eLi et al., 1993.

^fIdle et al., 1992.

paraoxonase (Li et al., 1993), sulphotransferase, UDP-glucuronosyltransferases, and *N*-methyltransferase (Idle et al., 1992). Paraoxonase metabolizes organophosphate pesticides such as paraoxon and chlorpyrifos. Interindividual variability in paraoxonase activity exceeds 100-fold in humans (Mutch et al., 1992). Despite the demonstrated difference in metabolic capabilities, paraoxonase activities have not yet been correlated with systemic toxicity. It is possible that the missing health outcome could be supplemented by an appropriate animal model where the relationship between exposure, biomarker, and health outcome is known. The level of paraoxonase activity differs substantially between species and may predict the degree of cholinesterase inhibition and systemic toxicity produced by exposure (Li et al., 1993). A model using this relationship could be used to estimate the risk to human populations based on their paraoxonase activities. While human health effects data are preferable, this strategy permits utilization of existing information.

4.6. Multiple susceptibility factors

Characterization of the variability in response to a contaminant requires the analysis of several susceptibility factors as demonstrated in the following example. Heterocyclic amines, which

cause colon cancer, are activated by the enzyme cytochrome P450 1A2 (CYP 1A2). After activation, the product undergoes NAT2-mediated conjugation. A recent case-control study of colorectal cancer patients found no differences in the proportions of the susceptible phenotypes for either CYP 1A2 or NAT2. When individuals having the susceptible forms of both enzymes were identified, they were 2.79 times more likely to be cases than controls (Lang et al., 1994). Thus, the risk for those having the more susceptible phenotype for both activating and conjugating enzymes exceeds the risk found in people who have the susceptible phenotype for only one of the enzymes.

Lead constitutes a rare example where the dose-response relationship has been thoroughly studied epidemiologically. Numerous studies have measured health outcome as a function of an intermediate endpoint, blood lead levels (BLLs). BLLs unambiguously identify the exposure, correspond to internal dose once steady-state is reached, and are highly correlated with adverse health outcomes.

Exposure to lead can produce reproductive, neurological, and cardiovascular damage (Goldstein, 1990; Preuss, 1993). Table 5 lists many of the susceptibility factors known to modify the risk of lead-induced toxicity.

Table 5
Susceptibility factors modifying the neurological effects of lead

Category	Susceptibility factor	Health outcome modified
Age	Prenatal ^a Early childhood: immature nervous system ^b , efficient GI absorption ^b , aerosol retention ^c	Neurological and neuromuscular damage
Gender	Boys ^d Girls ^e Women ^f	Recovery from neurological damage Neuropsychological effects BLL, systemic toxicity
Genetics	δ -ALA polymorphism ^g	BLL, systemic toxicity
Ethnicity	δ -ALA1 polymorphism ^h	BLL, systemic toxicity
Quality of life	Aggregate factors ^h SES status/dietary calcium ⁱ	BLL, systemic toxicity BLL, systemic toxicity

ALA, aminolevulinic acid; BLL, blood lead levels; SES, socioeconomic status.

^aBellinger et al., 1990.

^bPreuss, 1993.

^cDeRosa et al., 1991.

^dBellinger et al., 1990.

^eMcMichael et al., 1992.

^fSilbergeld et al., 1989.

^gWetmur et al., 1991.

^hBellinger et al., 1990.

ⁱMahaffey et al., 1986.

Early development is associated with susceptibility to lead-induced neurological damage. While neurotoxicity has been demonstrated in adults, the magnitude and types of toxicity are different. In children, lead exposure is manifested as impaired behavioral, cognitive, and motor function. As recently as 1991, BLLs below 25 $\mu\text{g}/\text{dl}$ were assumed to protect from neurological damage. Currently, deficits are thought to occur with BLLs below 10 $\mu\text{g}/\text{dl}$ with a calculated threshold of less than 1 $\mu\text{g}/\text{dl}$ (Schwartz, 1994). The size of this highly susceptible population is staggering. Between 3 and 4 million children between the ages of 0.5 to 5 years old have been estimated to have BLL levels greater than 15 $\mu\text{g}/\text{dl}$ (Crocetti et al., 1990).

Children are highly susceptible because of their developing nervous systems (Needleman and Gatsonis, 1990; Bellinger et al., 1991; Dietrich et al., 1993), greater gastrointestinal uptake (30-50% in children, 7-15% in adults) (Preuss,

1993), and high retention of inhaled aerosol (DeRosa et al., 1991).

Gender influences on susceptibility are age-linked. When controlling for childhood exposure, boys recover more slowly from the cognitive delays produced by prenatal exposure (Bellinger et al., 1990), while girls from age 2 to 4 appear to be more sensitive to neuropsychological effects than boys (McMichael et al., 1992). In women, menopause results in mobilization of bone lead stores which is detected as an increase in BLL in the absence of additional exposure (Silbergeld et al., 1989).

A genetic trait is relevant when considering the risk posed by lead exposure. δ -Aminolevulinic dehydratase is a polymorphic enzyme involved in heme biosynthesis. While both alleles, ALA1 and ALA2, produce enzymes that are inhibited by lead, the ALA2 enzyme is inhibited to a greater degree. Although reports have been inconsistent, ALA2 homozygotes tend to have higher blood

(Wetmur et al., 1991) or bone lead levels (Smith et al., 1995). Ethnicity is intertwined with this genetic risk factor. The frequency of the sensitive allele is 19% in whites and < 1% in African-Americans (Wetmur et al., 1991).

Potential susceptibility factors related to "quality of life" can be identified. Animal studies indicate that dietary inadequacies can produce augmented lead toxicity or tissue accumulation. Vitamin B6 deficiency (McGowan, 1989), iron deficiency (Hashmi et al., 1989), protein deficiency (Ghosh et al., 1992), and maternal zinc deficiency (Ashraf and Fosmire, 1985) have been linked to enhanced toxicity in animal but not human studies. Ominously, children from poor families are likely to consume diets deficient in calcium, iron, zinc, magnesium (Drake, 1992; Johnson et al., 1994). Although BLLs in children are known to be inversely correlated with dietary calcium levels (Mahaffey et al., 1986), the effect of other dietary deficiencies have not been epidemiologically determined.

Other susceptibility factors stemming from quality of life are difficult to assess due to the use of aggregate risk factors. Children from poorer homes are more severely affected by prenatal lead exposures than children from more affluent homes. In this case, the sociodemographic characteristics such as social class and income may serve as a surrogate for an unidentified susceptibility or exposure factor (Bellinger et al., 1990).

Despite extensive dose response information for some susceptibility factors, such as those relevant for childhood neurological effects, other modifiers of lead toxicity related to quality of life have not been studied in human populations.

5. Conclusion: implications for risk assessments

The failure to consider differences in susceptibility may result in the promulgation of standards that are not protective for highly susceptible segments of the population. The current ambient air standard for respirable particulate is an example where children and people with asthma continue to be affected at permissible ambient levels. Yet, measurement of only

the population response variability is usually not feasible and would do little to describe the populations at risk. What is critical for quantitative risk assessment is the identification of the factors contributing to susceptibility accompanied by data describing the relationship between exposure and illness for those factors.

Quantitative assessment of the risk to highly susceptible populations may not be possible because parts of the dose response relationships for the relevant susceptibility factor(s) are missing. What first appears to be a rich literature, such as that describing polymorphic detoxifying enzymes, may provide no usable dose-response relationships for the risk assessor. Inadequacies may be found in any of the constituents of the dose response relationship.

The options for incorporating susceptibility information into risk assessments depends on the nature of the missing data. Obviously, risk cannot be estimated for populations where the susceptibility factors have neither been identified nor evaluated. Also, risk assessments are contaminant specific and so cannot evaluate the impact of susceptibility factors where the exposure agent is unknown. Whether some risk factors measure aspects of susceptibility may be questionable, especially in situations where confounding due to unidentified exposure or other risk factors is likely. Risk factors due to ethnicity demonstrate that it may not be possible to disentangle the role of susceptibility from other contributors to risk.

When the available data does not support a quantitative risk assessment, qualitative risk assessments may be possible. Qualitative risk assessments describe the risk to a population based on, among other descriptors, their projected exposures, behavioral characteristics and geographic location. The size of the susceptible population is important for determining the impact of the risk. Over 1 million African-Americans are deficient in G6PD and a small decrement in function would have a great effect on morbidity (Amoruso et al., 1986). Qualitative risk assessments may be possible when the suspected susceptibility factor has not been thoroughly evaluated (as with dietary factors), when

health effects are known but exposure measurements are unavailable (as with G6PD deficiencies), and when biomarkers suggest the presence of an unexamined health outcome (as with diepoxybutane-induced SCEs).

Some incomplete dose-response data may be used in quantitative risk assessments if the missing health outcome information can be extrapolated from animal models. Susceptibility factors associated with biomarkers may fall into this category. Once validity of the animal model is established, the relationship between exposure and biomarker measured in human studies can be supplemented by animal models of the relationship between a biomarker and the adverse health outcome. This information can be used to estimate the risk of health effects in human population based on their biomarker response.

In the rare instances where multiple factors contributing to susceptibility have been identified, many are accompanied by significant data deficiencies. Several susceptibility factors were identified as influencing susceptibility to lead. However, the role of the dietary factors in determining susceptibility have not been confirmed and socioeconomic status is subject to confounding. Even though lead is a well-studied toxicant, the quantitative assessment of risk for susceptible populations will be based on only some of the known susceptibility factors. Most important, while there is evidence that many different factors may contribute to susceptibility to lead intoxication, their interaction has not been studied. Interactions between the ALA polymorphism, developmental stage, dietary deficiencies, and gender are possible. As a result, the the most susceptible population cannot be identified.

While many of the deficits in the data describing susceptibility may be unavoidable, epidemiological investigations can be designed to evaluate variability in susceptibility. Controlled-exposure studies can evaluate non-carcinogenic susceptibility factors. Studies can identify susceptibility factors by stratifying on the basis of response and examining the biological basis for the difference. Response variability can be analyzed in large cohorts with uniform exposure. Future approaches to risk assessments may em-

ploy mechanistic models where the variability in the intermediate steps between exposure and health outcome are modeled using a combination of animal and human, in vivo and in vitro endpoints.

Acknowledgements

The research for this article was performed during a 1994 AAAS Science and Engineering Fellowship at the U.S. Environmental Protection Agency. The assistance and guidance of Angela Nugent is gratefully acknowledged. The views expressed here are the author's and do not represent the policy of either the EPA or AAAS

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Evaluating human variability in chemical risk assessment: hazard identification and dose-response assessment for noncancer oral toxicity of trichloroethylene

H.A. Barton^{*a}, C.D. Flemming^a, J.C. Lipscomb^b

^aManTech Environmental Technology, Inc., P.O. Box 31009, TriService Toxicology, Wright-Patterson Air Force Base, Dayton, OH 45437, USA

^bToxicology Division, Armstrong Laboratory, OL AL HSCIOET Bldg 79, 2856 G. St., TriService Toxicology, Wright-Patterson Air Force Base, Dayton, OH 45433, USA

Abstract

Human variability can be addressed during each stage in the risk assessment of chemicals causing noncancer toxicities. Noncancer toxicities arising from oral exposure to trichloroethylene (TCE) are used in this paper as a case study for exploring strategies for identifying and incorporating information about human variability in the chemical specific hazard identification and dose-response assessment steps. Toxicity testing in laboratory rodents is the most commonly used method for hazard identification. By using animal models for sensitive populations, such as developing fetuses, testing can identify some potentially sensitive populations. A large variety of reproductive and developmental studies with TCE were reviewed. The results were mostly negative and the limited positive findings generally occurred at doses similar to those causing liver and kidney toxicity. Physiologically based pharmacokinetic modeling using Monte Carlo simulation is one method for evaluating human variability in the dose-response assessment. Three strategies for obtaining data describing this variability for TCE are discussed: (1) using in vivo human pharmacokinetic data for TCE and its metabolites, (2) studying metabolism in vitro, and (3) identifying the responsible enzymes and their variability. A review of important steps in the metabolic pathways for TCE describes known metabolic variabilities including genetic polymorphisms, enzyme induction, and disease states. A significant problem for incorporating data on pharmacokinetic variability is a lack of information on how it relates to alterations in toxicity. Response modeling is still largely limited to empirical methods due to the lack of knowledge about toxicodynamic processes. Empirical methods, such as reduction of the No-Observed-Adverse-Effect-Level or a Benchmark Dose by uncertainty factors, incorporate human variability only qualitatively by use of an uncertainty factor. As improved data and methods for biologically based dose-response assessment become available, use of quantitative information about variability will increase in the risk assessment of chemicals.

Keywords: Trichloroethylene; Risk assessment; Reproductive and developmental toxicity; Sensitive populations; Monte Carlo simulation

Presented at the "Conference on Risk Assessment Issues for Sensitive Human Populations", Hope Hotel and Conference Center, Wright-Patterson Air Force Base, OH, April 24-27, 1995.

*Corresponding author, ICF Kaiser, P.O. Box 14348, Research Triangle Park, NC 27709.

1. Introduction

During risk assessment of chemicals causing noncancer toxicities, the variability of human populations, including sensitive individuals, can be addressed in several ways. Toxicity testing in animal models for sensitive populations can identify potential hazards for which dose-response values, such as U.S. EPA's reference dose (RfD), would be required (see Table 1). Variations in exposure patterns for human populations can be described using Monte Carlo simulation methods, but this will not be addressed further as we will focus on chemical-specific issues. Increased use of biologically-based modeling methods for developing dose-response values can incorporate quantitative information about variability. Modern molecular and genetic techniques facilitate the identification and quantitation of toxicokinetic and toxicodynamic factors responsible for human variability including genetic polymorphisms, induction or repression of enzymes, disease states, and aging.

Dose-response assessment methods may be divided into those for estimating doses and those for relating the doses to the response. Methods

of dose estimation have included allometric scaling and physiologically based pharmacokinetic (PBPK) modeling. Response modeling has moved towards more biologically based methods, but this process is frequently hindered by lack of knowledge of the mode of action which leads to chemical toxicity. Therefore, default approaches have been used for evaluating the dose-response relationship, such as the No-Observed-Adverse-Effect-Level (NOAEL) or benchmark dose (BMD) divided by uncertainty factors (UF).

This paper is a case study of noncancer toxicities arising from oral exposure to trichloroethylene (TCE). Trichloroethylene is a widely used industrial chemical which, as a result, is a widespread contaminant of soil and groundwater. It has been the subject of much toxicity testing and research which has created one of the most extensive databases existing for an industrial chemical (Davidson and Beliles, 1991; ATSDR, 1993). Most of the chronic studies focused upon cancer endpoints, particularly mouse liver and lung tumors and rat kidney tumors; however, noncancer endpoints also have been extensively studied.

Both oral and inhalation exposures have been evaluated in risk assessments for TCE. Oral exposures are most often associated with consumption of water, while inhalation may occur during the use of water for activities such as showering or by exposure to TCE vapors in occupational settings. This case study focused on concerns about exposure via drinking water, although many TCE-induced toxicities in animals are similar regardless of the exposure route.

The remainder of this paper describes strategies for incorporating variability into the process of developing dose-response values for TCE. First, toxicity testing can begin to identify potentially sensitive populations. Secondly, we will describe the use of data on human pharmacokinetics and pharmacogenetics to provide insights into human variability that could be incorporated in pharmacokinetic modeling. Finally, experimental studies and interspecies comparisons can provide insight into variations in responsiveness that can help inform decision making about the use of uncertainty factors.

Table 1
Strategies for incorporating human variability in chemical risk assessment

Risk assessment step	Strategy
Hazard identification	Testing in animal models for potentially sensitive populations <i>In vitro</i> assays Toxicity testing with induced or coexposed animals
Exposure assessment	Monte Carlo simulation of population distributions of activities, ages, etc.
Dose-response assessment	Uncertainty factor for human variability Monte Carlo simulation of pharmacokinetic variability using PBPK modeling Incorporating variability in biologically based response modeling

2. Noncancer toxicity testing and identification of potentially sensitive populations

Hazard identification is the first step in the risk assessment process. It generally relies upon toxicity testing in laboratory animals to identify potentially sensitive populations. This can be done by mimicking the exposure routes of concern and by using animal models for specific sensitive populations (Amdur, 1989).

Studies of a wide range of toxic endpoints and biochemical changes have been reported using oral exposure to TCE (ATSDR, 1993). Most studies used corn oil gavage, which is a confounding factor responsible for altering the pharmacokinetics of TCE as compared to drinking water (Withey et al., 1983; Merrick et al., 1989) and for altering lipid metabolism and other pharmacodynamic processes. Few studies used water as the vehicle due to poor solubility of the compound, although some drinking water or water gavage studies have used emulsifying agents (Tucker et al., 1982). A few studies used microencapsulated TCE given in feed (NTP, 1985, 1986). Because inhalation studies do not utilize a vehicle, (comparison of inhalation and gavage results using PBPK) models can help separate out vehicle effects.

The best documented systemic effects from TCE exposure are neurotoxicity, hepatotoxicity, and nephrotoxicity in adult animals. Reproductive and developmental toxicity also have been extensively studied; the largely negative results are reviewed below. Except for neurological activity, there is limited documentation of effects in humans.

Central nervous system (CNS) depression resulting from exposures to high concentrations by inhalation has long been known, hence, the use of TCE as an anesthetic in humans. Oral data for neurotoxicity is very limited, particularly with low doses and even with animals (Isaacson and Taylor, 1989; Isaacson et al., 1990). Several strains of rats exposed to 500 and 1000 mg/kg/day in a lifetime corn oil gavage study had clinical signs of CNS toxicity including transient convulsions, although no effects had been observed in a 13-week study (NTP, 1988). Only a

few neurobehavioral measurements included in developmental studies gave positive results (NTP, 1986). Too little data are available on neurotoxicity associated with oral exposures, particularly at low concentrations, to evaluate how this effect might vary among different humans.

Hepatotoxicity has been associated with both oral and inhalation exposures to TCE, particularly in animals. Association of liver toxicity in humans with TCE exposure has been limited considering the extensive occupational and pharmacological exposures (Davidson and Beliles, 1991). In two year chronic studies, mice exposed to approximately 1000 to 2000 mg/kg/day by corn oil gavage developed liver tumors (ATSDR, 1993). No liver toxicity was reported in either of two chronic studies with four strains of rats dosed by corn oil gavage 500 and 1000 mg/kg/day (NCI, 1976; NTP, 1988). Short exposures (i.e. weeks) resulted in liver toxicity in mice as indicated by increased serum levels of liver enzymes (Buben and O'Flaherty, 1985) and histopathology (Elcombe et al., 1985). The most commonly reported liver change was increased liver to body weight ratio (fractional liver weight) at doses above 100 mg/kg/day. Generally, this has not been considered an adverse effect for risk assessment, however, it may be interpreted as a biomarker or early indicator of liver toxicity (Dourson, 1994; Barton and Das, 1996). Unfortunately, there is too little data to know how the variability between species compares to the variability of the human population for TCE-induced liver toxicity.

Kidney toxicity has rarely been reported in humans, though it is the major noncancer toxicity reported in lifetime oral dosing studies with both rats and mice (NCI, 1976; NTP, 1988). It is believed to result from the formation of activated metabolites of dichlorovinylglutathione which arise in a metabolic pathway beginning with conjugation of TCE with glutathione (Jaffe et al., 1984). This pathway has recently been shown to be present in humans.

Reproductive and developmental testing has become increasingly important because developing human fetuses and newborns are recognized

as sensitive populations for some chemicals. Developmental toxicity has been suggested to be associated with human exposures to drinking water containing chemical mixtures including TCE (Davidson and Beliles, 1991). The hypothesis suggested by the epidemiological studies, currently has only limited support from experimental studies of reproductive and developmental effects in rats, mice, and rabbits. A wide range of endpoints have been evaluated, including male and female reproductive success, teratogenicity, and effects in the developing neonates exposed in utero and/or post partum.

Both female and male reproductive function have been studied in mice, rats, and rabbits. Effects on implantation's, litter size, fetal resorptions or other similar measures of reproductive success were not observed following exposures in water, feed, or air (Schwetz et al., 1975; Dorfmueller et al., 1979; Hardin et al., 1981; NTP, 1985; Cosby and Dukelow, 1992; ATSDR, 1993; Dawson et al., 1993) except in two studies (Healy et al., 1982; NTP, 1986). Wistar rats exposed to 100 ppm TCE from days 8-21 of pregnancy had increased fetal resorption and decreased litter size (Healy et al., 1982). By contrast, exposure of Long-Evans rats to 1800 ppm TCE during pregnancy produced no effects on resorption sites, liver fetuses/litter, or fetal body weight (Dorfmueller et al., 1979). Rats fed microencapsulated TCE in their diet showed only small changes, characterized by the authors as resulting from generalized toxicity and not specific effects on the reproductive system (NTP, 1986). Predominantly negative results were reported for alterations in sperm morphology and sperm motility in mice, rats, and rabbits (Hardin et al., 1981; Land et al., 1981; NTP, 1985, 1986). Two NTP feeding studies reported increases in the organ weight ratio of the testicles and epididymis in the high dose group F₁ mice and rats (NTP, 1985, 1986). No effects on the reproductive function of male rats were reported except at a dose (1000 mg/kg) high enough to cause neurobehavioral effects (Zenick et al., 1984). These studies demonstrate that TCE is not a significant reproductive toxicant in these species.

Studies of developmental effects indicated that TCE also was not a potent developmental toxicant. Fetal or pup (day 0) body weights were unaffected in five studies (Schwetz et al., 1975; Dorfmueller et al., 1979; Beliles et al., 1980; NTP, 1986; Cosby and Dukelow, 1992) and decreased in two others (Healy et al., 1982; NTP, 1985). Small changes in the growth of offspring resulting from either exposure of the parental generation or exposure of both the parents and the developing young have also been reported (Dorfmueller et al., 1979; Manson et al., 1984; NTP, 1986).

Minor skeletal anomalies have been inconsistently reported. Incomplete ossification of the sternum or other bones in mice, rats, and rabbits at the end of pregnancy was described, though the results were not always statistically significant (Schwetz et al., 1975; Dorfmueller et al., 1979; Beliles et al., 1980; Healy et al., 1982). No skeletal anomalies were found in two inhalation studies with Sprague-Dawley rats (Schwetz et al., 1975; Beliles et al., 1980) or a corn oil gavage (250 mg/kg/day) exposure of B6D2F1 mice (Cosby and Dukelow, 1992).

Reports of soft tissue anomalies were rare. The study with Long-Evans rats exposed to 1800 ppm reported a statistically significant increase (displaced right ovaries); the effect was present in those exposed during pregnancy only but not in those exposed before and during pregnancy (Dorfmueller et al., 1979). External hydrocephalus in rabbits has been reported, but it was unclear if it was attributable to TCE exposure (Beliles et al., 1980). A dose-related increased incidence of eye abnormalities (reduced or absent eye bulges) has been reported recently in one and six day old pups born to F344 rats exposed to TCE by corn oil gavage (Narotsky et al., 1995). A minimal increase was reported at 320 mg/kg/day increasing up to 30% incidence at 1125 mg/kg/day.

Increased incidences of cardiac malformations have been reported recently (Dawson et al., 1990, 1993). TCE and dichloroethylene (DCE) caused heart defects in 21% and 14% of fetuses, respectively, as compared to control incidences of 3-4% upon direct placement of chemical in the uterus

of pregnant Sprague-Dawley rats (Dawson et al., 1990). This was followed by drinking water exposures of rats which showed about a 10% increase in cardiac malformations over the control incidence (Dawson et al., 1993). The significance of this work may be in doubt due to the similar small increase reported at both doses tested (differing by almost 400-fold) and because there was no clear relationship to the timing of the exposure to TCE (e.g. during pregnancy, prior to and during). However, the significance of the results may be supported by findings with TCA, a metabolite of TCE, and by epidemiological studies. TCA caused cardiac abnormalities when Long-Evans rats were dosed by aqueous gavage (Smith et al., 1989). These TCA exposures used bolus dosing with concentrations much higher than the TCE given in the drinking water study. An epidemiological study reported increased incidences of cardiac anomalies in children born to mothers drinking water containing TCE and DCE (Goldberg et al., 1990). Identification of the mechanism responsible for the cardiac anomalies would clarify the link to chemical exposures.

Limited developmentally related neurologic or neurobehavioral changes occurred with TCE exposure in rats and mice. Exposures included various combinations of times prior to, during, and post-gestation. Small decreases in myelination were reported (Isaacson and Taylor, 1989). These biochemical changes might be anticipated to cause behavioral alterations, but studies of the behavior of F₁ generation animals were not conclusive. The NTP two-generational study of mice found a transient alteration in one measure of behavior in an open field test among F₁ mice, but not in any of the other measures (NTP, 1986). Inhalation exposure prior to and during gestation had no effect in tests of general activity levels in F₁ rats (Dorfmueller et al., 1979). TCE exposure through drinking water until 21 days after birth was reported to increase exploratory behavior in 60- and 90-day old males (Taylor et al., 1985). Increased activity on the running wheel was also reported at 60 days. No consistent pattern is evident from these results, so addi-

tional research is required to clearly demonstrate effects due to TCE.

Overall, the large number of studies in several species using various exposure routes and durations suggest that pregnant women and developing fetuses and infants are unlikely to be particularly sensitive to TCE. Trichloroethylene exposure caused limited reproductive or developmental toxicity even at doses that caused kidney or liver toxicity in chronically exposed adult animals. Thus, limiting exposures based upon toxicity's in other organs should be adequate to prevent reproductive or developmental effects.

3. Incorporating human variability into dose modeling

Most of the toxicities of TCE are believed to be due to its metabolites, so description of the pharmacokinetic variability for the parent and metabolites is important. Incorporation of this metabolic information into the development of dose-response values is best done using physiologically based pharmacokinetic (PBPK) models. Sensitivity analyses of PBPK models for organics have shown that estimates of metabolic rates are particularly important (Clewell et al., 1994). A few physiological parameters, such as ventilation rates, are also important but since they are not chemical specific they will not be discussed further (Hattis and Silver, 1994). Efforts are underway in several laboratories to expand existing models for the parent compound to incorporate metabolites. One strategy is to link models for TCE and its metabolites to describe the pharmacokinetics occurring after dosing with each compound (Clewell et al., 1995). This would facilitate integration of the substantial toxicity information available for TCE and each metabolite.

The major steps in the metabolic pathway for TCE are illustrated in Fig. 1. Two pathways begin with TCE, oxidation by cytochromes P450 and conjugation with glutathione. The glutathione transferase catalyzed conjugation initiates a minor pathway that forms dichlorovinylglutathione and subsequent metabolites. The

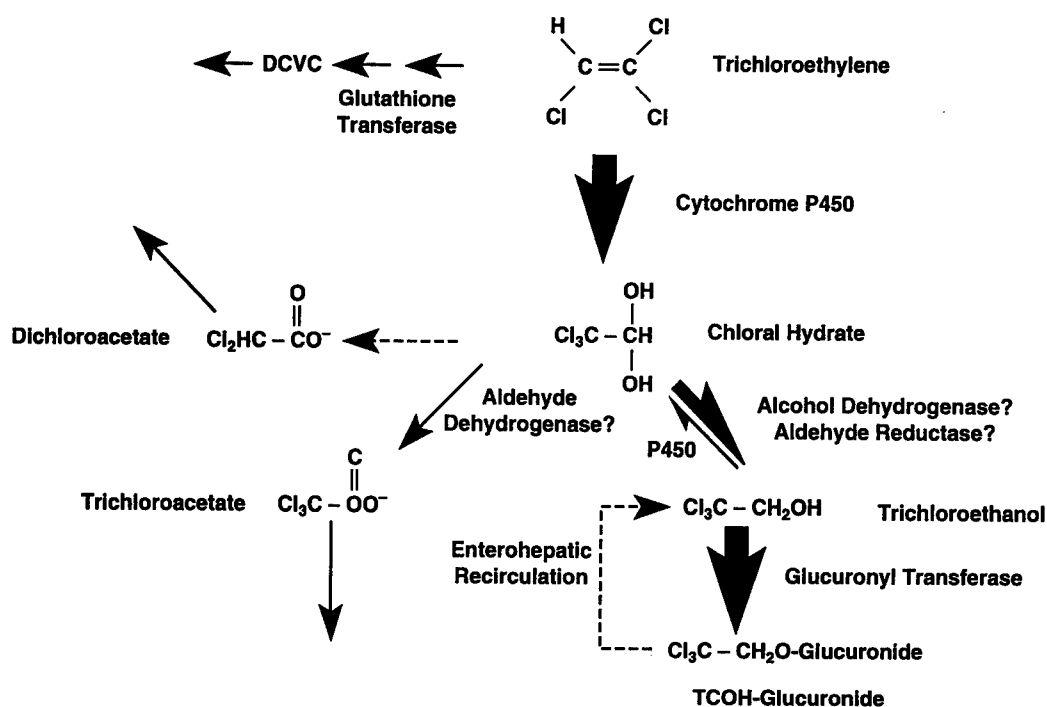


Fig. 1. Metabolic pathways for trichloroethylene and its metabolites. Enzymes that can catalyze the reactions are indicated.

major pathway, mediated by P450, results in the formation of trichloroacetaldehyde (chloral) which spontaneously hydrates to form chloral hydrate (CH). In vitro studies with microsomes support the rearrangement of an iron-oxygen-TCE complex in the P450 active site releasing predominantly chloral and only small amounts of trichloroethylene epoxide (Miller and Guengerich, 1982). This appears to be a critical factor distinguishing TCE from its mutagenic and genotoxic monochlorinated analog, vinyl chloride (Clewell et al., 1995). A second split in the metabolic pathways occurs with the metabolism of CH. Oxidation of CH forms trichloroacetate (TCA), while reduction produces trichloroethanol (TCOH). The major pathway is formation of TCOH followed by its glucuronidation. The glucuronide conjugate and TCA are the major urinary metabolites in all species studied. Several other minor metabolites have also been identified including dichloroacetate (DCA), monochloroacetate, and oxalic acid (Lipscomb et

al., 1995). These reactions convert the volatile, lipophilic parent into nonvolatile water soluble compounds that are readily excreted.

Variability is frequently incorporated into PBPK modeling using Monte Carlo simulation. This method uses a description of the distribution of values for a parameter (e.g. a normal distribution around the average body weight). Random sampling of the distribution produces a set of values that can then be sequentially used in the PBPK model to determine the variation in the output, usually a potential dose-metric for use in subsequent dose-response modeling.

Descriptions of pharmacokinetic variability in human populations could potentially be obtained from several sources to provide the data needed for Monte Carlo simulation and PBPK modeling. Human pharmacokinetic data for TCE and several metabolites could be utilized. Alternatively, estimates of the variability of human metabolism could be obtained from in vitro studies using human tissues. Finally, descriptions of

the variability of the enzymes involved in each metabolic step could be used in the PBPK model. Available data from each of these sources is reviewed below.

3.1. Human pharmacokinetic data

Human pharmacokinetic data are available for TCE, CH, TCOH, TCA, and DCA due to the pharmacological uses of most of these compounds (Wells et al., 1980; Allen and Fisher, 1993). Generally these studies used only a few healthy adult subjects so there is little direct information on variability, except with CH exposure. Studies with CH have focused on three populations, healthy adults, adults drinking ethanol, and children.

Marshall and Owens (1954) described metabolism of CH in up to 18 individuals whose age, sex, and health status were not specified. Individuals were dosed for 5 to 20 days and the percent of CH oxidized to TCA was estimated once steady-state apparently had been reached. Oxidation to TCA was normally distributed in this population as determined by the Wilk-Shapiro method (SAS Institute Inc., 1990) with a mean and standard deviation of $25 \pm 9\%$. The maximum was 47%, while the minimum was 5%, suggesting 10-fold variability in this process. These data indicate 53 to 95% of CH was reduced to TCOH, but due to the conversion of TCOH to TCA, these are minimum estimates of CH reduction. A later study indicates that there is also some daily variability for any single individual which under conditions approximating steady state appears to be less than 2-fold (Owens and Marshall, 1955). Limited information on variability was obtained from estimates made in seven healthy males of the half-life of TCA produced from CH. They ranged from 42 to 110 h with a mean of 75.3 but no further data are reported (Sellers et al., 1978). These estimates of variability do not address the contributions of measurement error or limited sample size to the apparent variability (Hattis and Silver, 1994).

Coexposure to CH and ethanol have long been known to have greater clinical effects than exposure to CH alone (Sellers et al., 1972). Studies in limited numbers of healthy adults showed altered

pharmacokinetics for TCOH and TCA, as well as for ethanol and its metabolites during coexposures. These studies are consistent with animal data indicating that metabolism of ethanol increases formation of TCOH from CH due to increased formation of alcohol dehydrogenase complexed with NADH (Shultz and Weiner, 1979). People receiving coexposures of TCE and ethanol may represent a sensitive population (due to metabolism through CH) though this has not been clearly demonstrated.

Chloral hydrate has been used for sedation of infants and children, including those born prematurely so pharmacokinetic studies have been reported (Gorecki et al., 1990; Mayers et al., 1991). One study with 22 subjects suggested that the half life of TCOH was more variable among preterm infants compared to full term infants or young children. These differences may result from alterations in metabolism as the child develops, such as changes in glucuronosyl transferase activity, or other effects, such as enzyme saturation.

3.2. *In vitro* data

Direct measurements of the variability of the metabolism of TCE and its metabolites could be made *in vitro*, but currently, no published data are available. An 8-fold variation in the V_{\max} for TCE has been found with microsomes from more than 25 humans (Lipscomb et al., submitted).

3.3. Variability of metabolizing enzymes

Data describing the variability of pharmacokinetically important factors such as enzymes, serum binding protein levels, or transport protein could be used to quantitate variability in humans, regardless of the chemical with which they were obtained. Recently, this approach was used with a PBPK model for 4-aminobiphenyl (Bois et al., 1995). Data on the variability of some enzymatic activities were available for 4-aminobiphenyl as the substrate, but, often data for the specific isoforms involved in the metabolism of 4-aminobiphenyl were unavailable. Therefore, the authors relied upon any data for variability of any isoform of the enzyme family of interest.

3.3.1. Trichloroethylene to chloral hydrate

Oxidation of TCE to trichloroacetaldehyde is catalyzed by the cytochromes P450. Trichloroacetaldehyde is unstable in aqueous solution and rapidly hydrates to chloral hydrate (CH) (a dihydric alcohol). Data from rat liver preparations demonstrated that several P450s metabolize TCE including members of the 1A, 2B, 2C, and 2E subfamilies (Nakajima et al., 1992). P450 2E1, which is well conserved across mammalian species, shows a preference (low K_m) for low-molecular-weight compounds including ethanol and many volatile organic solvents. Currently, the extent of involvement of human isoforms other than 2E1 is unknown.

Interindividual variability of the activity of P450 2E1 has been described in liver microsome samples from several populations. Peter et al. (1990) reported kinetic constants for 2E1-specific chlorzoxazone (CZ) hydroxylation (V_{max} and K_m) and *N*-nitrosodimethylamine *N*-demethylation (NMDA) (approximate V_{max} for formaldehyde formation) in 14 unspecified human liver samples. Based upon the Wilk-Shapiro test, the results for the kinetic constants were normally distributed. The coefficient of variance for both V_{max} estimates was 45% (3.5 ± 1.5 nmol CZ-OH nmol^{-1} 2E1 min^{-1} and 1.2 ± 0.5 nmol mg^{-1} microsomal protein min^{-1} NMDA) and 18% for the K_m estimates (38.9 ± 6.9 μM). By contrast, total P450 and immunologically estimated 2E1 levels were lognormally distributed in this same population. Measurements of total P450 and CZ activity have also been reported in 54 human liver samples (Fig. 2) (IIAM, 1995). These data also fit a lognormal distribution. Total P450 (nmol/mg microsomal protein) had a geometric mean and standard deviation of 0.38 ± 0.09 , while CZ hydroxylation ($\text{pmol mg}^{-1} \text{min}^{-1}$) had a geometric mean and standard deviation of 860 ± 58 . As more samples are reported, analysis of individual subpopulations will become possible.

The mean total P450 content (nmol/mg microsomal protein) in liver microsomes from Caucasians (0.43 nmol/mg) was about 1.5-fold higher than in Japanese liver (0.26 nmol/mg) samples (Shimada et al., 1994). Metabolism of 7-eth-

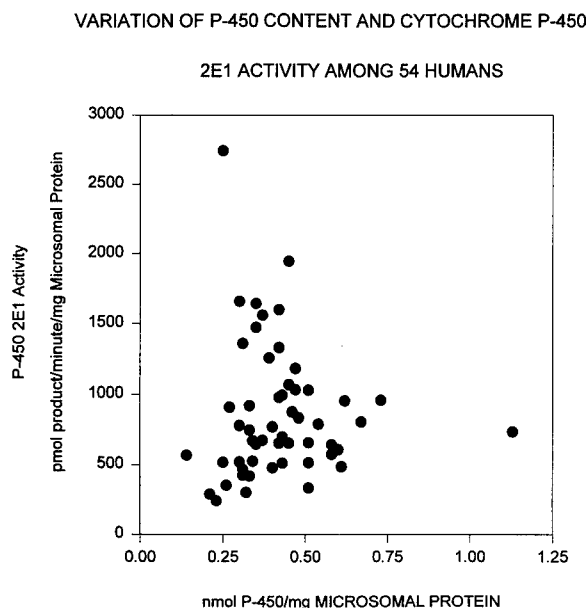


Fig. 2. Graph of the total cytochrome P450 and 2E1 isoform measured in microsomes from human livers showing the general lack of correlation. Data generously provided by the International Institute for the Advancement of Medicine (IIAM, 1995).

oxycoumarin was positively correlated (0.84) with immunological measurements of P450 2E1. Mean 7-ethoxycoumarin activity (per mg protein) in male Caucasians and Japanese differed by 1.5-fold as did the total P450. No marked gender-related differences were observed in either population. P450 2E1 quantitated immunologically varied nearly 10-fold among individuals. This isoform accounted for an average of 6.6 ± 2.9 (mean \pm S.D.) percent of total P450 present. The percent of total P450 accounted for by 2E1 was not affected by gender or age (12 to 73 years), nor were differences in 2E1-dependent metabolism. The summary form in which the data is presented in this paper does not allow determination of the distribution or other information required for Monte-Carlo simulation in a PBPK model.

Polymorphisms exist in the P450 2E1 gene, but have not been demonstrated to alter its enzymatic activity. Restriction fragment length polymorphisms have been identified using enzymatic digestion with *DraI*, *TaqI*, *RsaI*, *PstI* and *MspI*

(Stephens et al., 1994). Examination of allelic frequency in ethnic populations (European Americans, African Americans and Taiwanese) has revealed that certain alleles are positively correlated with protection from lung cancer and that these alleles are differentially distributed among these populations (Uematsu et al., 1991). However, in the absence of data relating these polymorphisms to enzymatic activity their implications for PBPK modeling of TCE and its metabolites remain unclear.

Ethanol ingestion, fasting/starvation, and diabetes result in induction of P450 2E1 in rodents (Sato and Nakajima, 1985). Increased 2E1 activity results in increased toxicity of TCE, so people with these conditions may represent sensitive populations (Nakajima et al., 1988). Predicting the effects of chronic exposure to ethanol is not straightforward. Ethanol results in the induction of 2E1, but it is also metabolized by P450 2E1 so the individual chronically exposed to ethanol may show increased activity of this isoform, but the individual co-exposed to ethanol and a second substrate for P450 2E1 may show decreased metabolic conversion of either or both compounds due to competitive inhibition. Ethanol also affects the activity of other enzymes in this metabolic pathway (e.g. alcohol dehydrogenase) so it may represent a major contributor to variability in TCE metabolic pathways.

3.3.2. Trichloroethylene to dichlorovinylcysteine

Conjugation of TCE with the ubiquitous cellular nucleophile, glutathione, is the initial step in the formation of dichlorovinylcysteine (DCVC). The reaction is catalyzed by the glutathione S-transferases (GST), a family of dimeric proteins, mostly cytosolic in origin, which are present in all mammalian species. Glutathione conjugates are further metabolized to yield cysteine derivatives which are excreted in urine. Generally, the products of this conjugation pathway are less toxic than the parent, but in some instances, this pathway leads to more toxic metabolites, such as the nephrotoxin, DCVC (Jaffe et al., 1984).

GST Theta class enzymes have been shown to form conjugates with a number of chlorinated low molecular-weight compounds (e.g. methyl

halides, methylene chloride) (Thier et al., 1991). While the GST Theta enzymes have not been conclusively demonstrated to be the enzymes responsible for glutathione conjugation of TCE, it seems reasonable to assume this given the general characteristics of the identified substrates. At least two Theta class GSTs have been isolated in the human, one form in liver and another in erythrocytes (Schroeder et al., 1995).

The distribution of transferase Theta enzyme and gene has been evaluated. It is present in 60 to 75% of the human population and absent in the remainder (Peter et al., 1989; Pemble et al., 1994). Hallier et al. (1993) correlated detoxification with GST Theta levels in whole blood taken from individuals classified as conjugators and non-conjugators. Blood exposed to methyl bromide, ethylene oxide or methylene chloride demonstrated no increase in the occurrence of sister chromatid exchange as compared to pre-exposure when obtained from conjugators. Blood of non-conjugators demonstrated a significantly increased degree of sister chromatid exchange post-exposure.

3.3.3. Interconversion of chloral hydrate and trichloroethanol

Identification of the enzymes involved in oxidations or reductions of aldehydes classically relied upon nicotinamide cofactor (NADH and NADPH) identification, patterns of inhibition, and partial or complete purification. Studies in rat liver and brain homogenates and subcellular fractions demonstrated that cytosolic NADPH-dependent aldehyde reductases and NAD-dependent alcohol dehydrogenases were capable of reducing CH to TCOH (Friedman and Cooper, 1960; Tabakoff et al., 1974; Ikeda et al., 1980, 1981; Ogino et al., 1990). The combination of ethanol and CH resulted in changes in the metabolism of both compounds (Sellers et al., 1972; Shultz and Weiner, 1979). Shultz and Weiner (1979) proposed that oxidation of ethanol by alcohol dehydrogenase formed an NADH-alcohol dehydrogenase complex that then reduced CH to TCOH. These data suggest that the extent

of involvement of each enzyme in rat is dependent upon the cofactor balance and metabolism of endogenous alcohols and aldehydes in the cells. Normal redox status of the liver results in cofactor concentrations of approximately NAD^+ (0.8 mM), NADPH (0.4 mM), NADH (0.2 mM), and NADP^+ (0.1 mM) (Hara et al., 1991), which would appear to favor the aldehyde reductases as the major enzymes contributing to CH reduction.

Another enzyme potentially involved in CH metabolism, at least in lung, is carbonyl reductase (Hara et al., 1991). This enzyme utilizes $\text{NADPH} > \text{NADH}$ to catalyze reduction to alcohols, but it can also oxidize secondary alcohols and chloral hydrate. Like alcohol dehydrogenase, release of the nicotinamide cofactor from this enzyme is slow, so cycling can occur. In this case the cycling would take one CH molecule to TCOH, followed by conversion of a second CH to TCA returning the cofactor to its reduced state.

No information was located describing the variability of aldehyde reductases or carbonyl reductase in human populations. By contrast, polymorphisms of several alcohol dehydrogenase genes are known (Agarwal and Goedde, 1992; Edman and Maret, 1992). Some 50% of Oriental populations have an inactive enzyme that is not found in Caucasian or Negroid populations. These polymorphisms may be important in chloral hydrate metabolism.

3.3.4. Metabolism of chloral hydrate to trichloroacetate

The variability of the formation of TCA from CH results from not only direct variation in this step, but also indirectly, from variation in the competing metabolic steps. Metabolism of CH is dependent upon two forward pathways, oxidation to TCA and reduction to TCOH, and the back reaction forming CH from TCOH (Fig. 1). Several papers report the formation of TCA from TCOH, but this may represent the back reaction ($\text{TCOH} \rightleftharpoons \text{CH} \rightleftharpoons \text{TCA}$) rather than an independent metabolic pathway catalyzing the two oxidations required for the conversion. Enterohepatic recirculation of TCOH-glucuronide re-

forming TCOH and alterations in serum binding of TCOH and TCA could also affect the apparent variability in the formation of TCA. Finally, variability of the elimination of TCA would alter its pharmacokinetics.

The enzyme(s) most likely involved in this step are the aldehyde dehydrogenases (ALDH) (Sladek et al., 1989). Alternatively, the potential involvement of cytochromes P450 is suggested by their ability to metabolize several cyclic aldehydes to carboxylic acids (Watanabe et al., 1990, 1991). However, subcellular fractionation studies found that microsomes were not the fraction responsible for the majority of metabolism of CH oxidation (Ikeda et al., 1980).

Mammalian ALDH are a multienzyme family that are located in the cytosol, mitochondria, and endoplasmic reticulum of many tissues and use NAD^+ as a cofactor. Individual enzymes have overlapping substrate specificities. Mouse liver contains eleven ALDH and several others are found only in other tissues (Dockham et al., 1992). Rat liver contains at least five isozymes while human livers have at least seven ALDH with varying substrate specificities (Dockham et al., 1992). Genes for four human ALDH have been characterized and polymorphisms have been identified for at least two (Yoshida, 1992).

Contradictory results have been reported for the role of ALDH in CH oxidation. Early studies by Cooper and Friedman (1958) reported CH oxidation to be a cytosolic activity. Cytosol and mitochondria from rat may both be major contributors to CH metabolism (Ikeda et al., 1980). This subcellular distribution appears consistent with that reported for ALDH: 34% mitochondria, 37% cytoplasm, and 10% microsomes, and 19% $500 \times g$ pellet (Crow et al., 1974). Cytosolic rates were up to seven times faster in the presence of NAD^+ than NADP^+ . The specific activity of the mitochondrial fraction was almost ten times that of cytosol and was dependent upon NAD^+ , but not NADP^+ . These characteristics are very similar to those reported for other reactions catalyzed by ALDH. In contrast, Sharpe and Carter (1993) report that CH is not metabolized by cytosolic and mitochondrial rat liver ALDH

as measured by conversion of NAD^+ to NADH. They compared propionaldehyde, monochloroacetaldehyde, dichloroacetaldehyde, and trichloroacetaldehyde metabolism in vitro and found monochloroacetaldehyde was metabolized at the highest rate, followed by propionaldehyde and dichloroacetaldehyde.

Inhibition of several ALDH by CH has been reported by investigators using nonchlorinated substrates. Chloral hydrate was a potent inhibitor of ALDH activity in both cytosol and mitochondria using D,L-glyceraldehyde as substrate (Crow et al., 1974). Oxidation of aldophosphamide by a semipurified human ALDH was noncompetitively inhibited by CH suggesting it did not bind to the active site of this enzyme (Dockham et al., 1992). Two other semipurified ALDH were far less well inhibited. It is possible that for some ALDH isoforms, CH is a relatively poor substrate and thus appears to be an inhibitor of much better substrates, while for other ALDH it may not be a substrate.

The involvement of aldehyde dehydrogenase is interesting from the perspective of human variability because polymorphisms of several genes (ALDH1, ALDH2, ALDH5) have been identified (Yoshida, 1992). Several variants of ALDH1 exist, including a form with severely reduced enzyme activity. Mitochondrial ALDH2 activity is lacking in approximately 50% of the Oriental population, but is present in virtually all Caucasians and North and South American Indians studied (Agarwal and Goedde, 1992). Multiple alleles of the ADH5 gene exist in Caucasians and Orientals, but the genomic analysis has not yet been extended to determining the properties of the resulting enzymes.

Identification of the specific enzymes involved in the oxidation of CH will improve our ability to address human variability in this step. Because TCOH, the reductive metabolite, is considered the active metabolite causing sedation, polymorphisms of TCA formation might not have become apparent in the clinical use of CH. However, they would be important for understanding the variability of TCA formation and its potential for toxicity.

3.3.5. Metabolism of trichloroethanol to chloral hydrate and/or trichloroacetate

Trichloroethanol has been considered to be the pharmacologically active sedative due, in part, to its prolonged presence in plasma as compared to CH which is often virtually undetectable. Therefore, exposures to TCOH were carried out in the studies of human CH pharmacokinetics (Marshall and Owens, 1954; Owens and Marshall, 1955). TCA was formed as a product, as has also been shown in rodents, however, there is insufficient information to describe the variability of this step.

Reduction of CH to TCOH is sometimes described as a reversible reaction. While this is thermodynamically true, it appears likely that different enzymes are involved in each direction. Friedman and Cooper (1960) reported that they were unable to oxidize TCOH in vitro using rabbit liver alcohol dehydrogenase even when they used extreme reaction conditions. Similarly, Sellers et al. (1972) reported that they were unable to oxidize TCOH to CH in vitro using purified horse liver alcohol dehydrogenase or rat liver supernatant. Pulmonary carbonyl reductase also does not catalyze oxidation of TCOH (Hara et al., 1991).

Although there is no direct data showing how TCOH is converted to TCA, the cytochromes P450 have been shown to metabolize the fluorinated analog of TCOH, trifluoroethanol, to trifluoroacetaldehyde (Kaminisky et al., 1992). Whether this is applicable to the chlorinated analogs remains to be determined.

3.3.6. Trichloroethanol glucuronidation

These enzymes exist as a family of microsomal isoforms in the rat, mouse and human. The isoforms themselves exhibit somewhat overlapping substrate activities, but other substrates are isoform-specific. The lack of enzyme activity is associated with severe metabolic disorders, namely Crigler-Najjar (C-N) syndrome and Gilbert's syndrome. Gilbert's syndrome occurs in 6% of the human population and results from reduced bilirubin-UDPGT activity (which may be dependent on more than a single UDPGT

isoform). A more severe condition known as C-N syndrome affects bilirubin glucuronidation as well. C-N Type I patients have a total absence of UDPGT activity and prognosis is poor. C-N Type II patients have extremely low bilirubin glucuronidation capacity, but can be treated with phenobarbital to induce enzyme activity. It is thought that C-N Type II and Gilbert's represent the homozygous and heterozygous condition, respectively (Ritter et al., 1991).

Inducers of UDPGT activity include treatment with drugs (e.g. phenobarbital, phenytoin, dexamethasone, oral contraceptives), ethanol consumption, and cigarette smoking (Bock et al., 1994). Glucuronidation is an important elimination pathway for a large number of drugs and interaction at this point in the pathway is a major pharmacological consideration. The dietary intake of cabbage and Brussels sprouts results in the induction of UDPGT, as these vegetables contain compounds which are similar to PAH-like inducers.

The identities of the UDPGT isoforms responsible for conjugation of TCOH have not been determined. Thus, no quantitative information is available to describe the variability of this enzyme. Furthermore, it is unclear if there are interactions between the conjugation of TCOH and either drugs or endogenous substrates. Because TCOH-conjugation appears to be a critical detoxification step, people in whom this might be impaired might be more susceptible to TCE toxicity.

3.4. Summary of pharmacokinetic variability

Several strategies for identifying potentially sensitive populations and quantitatively describing pharmacokinetic variability have been discussed. There is qualitative information that might be used to suggest specific populations which could be at increased risk. However, there are serious limitations in both the qualitative and quantitative data and methods for analyzing data. The identity of the isoforms involved in the metabolism of TCE and its metabolites is one major gap. Perhaps the most significant limita-

tion, however, is a lack of data relating pharmacokinetic variability to alterations in toxicity.

4. Variability in response modeling

In general, response or pharmacodynamic modeling is less well developed than is pharmacokinetic modeling. This reflects the much greater knowledge about the molecular details of pharmacokinetics processes than exists about molecular processes leading to toxicity. As a result, most dose-response modeling has been empirical, although there have been increasing efforts to develop biologically based methods.

Recently alternative methods for noncancer risk assessment have been proposed that try to utilize more of the available dose-response data rather than just using the dose considered a No-Observed-Adverse-Effect-Level. These approaches include the benchmark dose (Allen et al., 1994) and categorical regression (Hertzberg and Miller, 1985) methods. Approaches using statistical curve fitting may provide a framework within which to evaluate pharmacodynamic variability by analyzing multiple datasets. However, surprisingly few adequate datasets are available for TCE-induced noncancer toxicity (Barton and Das, submitted).

The modes of action for TCE-induced noncancer effects have not been identified so there is little that can be used to consider variability in pharmacodynamics. One indication of potential variability is the finding that preexposure of rats to ethanol or phenobarbital leads to hepatotoxicity (Nakajima et al., 1988; Okino et al., 1991). Rats exposed only to TCE do not develop hepatotoxicity. The role of ethanol in altering metabolism has been previously discussed and it is possible that such pharmacokinetic effects are responsible for the toxicity in rats. However, it is also possible that these compounds alter the pharmacodynamic processes in the liver. Chloroform, for instance, increases the liver toxicity of carbon tetrachloride by suppressing liver regeneration (Dalu and Mehendale, 1996). Further studies of the processes of liver and kidney re-

sponses to damage will be important for improved pharmacodynamic modeling and determining human variability.

5. Use of uncertainty factors

Development of dose-response values for non-cancer effects has largely relied upon the traditional uncertainty (safety) factor approach to convert empirical evidence into acceptable exposure levels for human populations. Variability is addressed by the application of the uncertainty factors (Dourson, 1994). One factor is explicitly for the variability of the human population. Indirectly, application of the uncertainty factor for interspecies extrapolation (e.g. the assumption that humans are more sensitive than the tested animal species) also may address issues of human variability. Testing chemicals in a relatively wide range of animals (i.e. not just rodents) can reflect the kind of variability that is seen in the human population (Amdur, 1989). Such data will, of course, reflect variation in both the pharmacokinetic and the pharmacodynamic processes. Some data is available for TCE that indicates, for instance, that the mouse is particularly sensitive to increases in fractional liver weight (Kjellstrand et al., 1981; Davidson and Beliles, 1991). Therefore, assuming that average humans are more sensitive than mice and that some humans are even more sensitive than the average may not be appropriate.

Throughout this paper we have identified several populations that may be at greater risk for TCE-induced toxicity. Identification of sensitive populations is largely speculative due to lack of data, even for a chemical as well studied as TCE. Because the use of uncertainty factors is driven in large part by this lack of sufficient data, there will always be a significant policy aspect to addressing sensitive populations in the development of noncancer dose-response values.

6. Conclusions

Strategies are developing for identifying human variability and incorporating it into the

development of dose-response values (Hattis and Silver, 1994). Toxicity testing for a broader range of endpoints plays a role in recognizing potentially sensitive populations, such as the developing young either before or after birth. Knowledge about the metabolic pathways and other factors influencing pharmacokinetics (e.g. binding proteins, transport proteins) can be very helpful. It can be combined with our increasing knowledge of the variability of these factors in humans to model variability using techniques such as Monte-Carlo simulation in PBPK modeling. A significant limitation however, is the current lack of knowledge on how variability in TCE pharmacokinetics influences alterations in toxicity. As knowledge of pharmacodynamic processes grows and biologically based modeling becomes an increasing reality, it will become possible to incorporate variability into this process as well.

As efforts are made to incorporate a broader range of scientific information into the development of dose-response values and other aspects of risk assessment, it is critical to have available as broad a range of methods as possible. Using a single method, such as the NOAEL/UF approach, provides a certain level of consistency that is valuable. However, it can also obscure very real differences between chemicals that fall outside the range of factors directly considered in this method. For instance, the NOAEL is frequently discussed as a level at which there is no risk, yet several analyses have shown that the risk of an effect at the NOAEL is variable and dependent upon the experimental design (Allen et al., 1994). Thus, while the method is consistent, the degree of protectiveness of the dose-response values derived using this method is not equal for all chemicals.

Other methods similarly have both strengths and weaknesses. When sufficient dose-response data are available, it may be preferable to use the benchmark dose approach rather than the NOAEL (Barton and Das, submitted). Physiologically based pharmacokinetic modeling can be combined with the NOAEL approach in some cases to better incorporate scientific data when it is available. The database for each toxic effect

and each chemical will vary, so having a range of usable methods can customize the development of the dose-response value to incorporate the greatest amount of scientific information while protecting public health.

Acknowledgments

This work was performed in part under Department of the Air Force Contract F33615-90-C-0532 with funding provided in part by the Strategic Environmental Research and Development Program.

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Toxicology 111 (1996) 289–303

TOXICOLOGY

Investigation of the impact of pharmacokinetic variability and uncertainty on risks predicted with a pharmacokinetic model for chloroform

Bruce C. Allen, Tammie R. Covington, Harvey J. Clewell*

K.S. Crump Group, ICF Kaiser International, 602 E. Georgia Avenue, Ruston, LA 71270, USA

Abstract

A sensitivity and uncertainty analysis was performed on the Reitz et al. (Toxicol. Appl. Pharmacol., 1990: 105, 443) physiologically based pharmacokinetic (PBPK) risk assessment model for chloroform. The analytical approach attempted to separately consider the impacts of interindividual variability and parameter uncertainty on the predicted values of the dose metrics in the model, as well as on liver cancer risk estimates obtained with the model. An important feature of the analytical approach was that an attempt was made to incorporate information on correlation between important parameters, for example, the observed correlation between total blood flow and alveolar ventilation rate. Using the published PBPK model for chloroform, the best estimate of the average population risk based on the preferred pharmacodynamic dose metric (PTDEAD), representing cell death, is 9.2×10^{-7} ; this estimate is more than 500-fold lower than the risk estimate of 5.3×10^{-4} based on an alternative pharmacokinetic dose metric (AVEMMB), which represents tissue adduct formation. However, when interindividual variability was considered the range of individual risks (from the 5th to the 95th percentile of the population) predicted with PTDEAD was extremely broad (from 3.0×10^{-13} to 3.2×10^{-4}), while individual risks predicted with AVEMMB only varied over a factor of four (from 1.9×10^{-4} to 7.4×10^{-4}). As a result, the upper 95th percentile of the distribution of individual risk estimates based on the preferred cell death metric were within a factor of three of the 95th percentile for the pharmacokinetic alternative. The crucial factor with respect to the much greater variability of chloroform risk estimates based on cell death is that the dose metric, PTDEAD, is exquisitely sensitive to variation of the parameters in the model defining the response of cells to the cytotoxicity of chloroform. Unfortunately, these key parameters are also highly uncertain, as well as strongly correlated. As a result it proved impossible to accurately quantify the additional impact of parameter uncertainty on the dose metrics and risk estimates for chloroform. In general, however, the approach used in this study should be useful for differentiating the impact of interindividual variability and parameter uncertainty on PBPK-based risk assessments of other chemicals where the sensitivity, uncertainty, and correlation of the key parameters are more limited.

Keywords: Pharmacokinetic modeling; Risk assessment; Uncertainty; Variability; Chloroform

*Corresponding author.

1. Introduction

Chloroform, which is formed in drinking water treated with chlorine (USEPA, 1985), has been observed to induce liver and kidney toxicity at relatively high concentrations (USEPA, 1985). Several chronic studies have also demonstrated that chloroform exposure increased the incidence of liver and kidney tumors in mice and rats (Eschenbrenner and Miller, 1945; NCI, 1976; Heywood et al., 1979; Palmer et al., 1979; Roe et al., 1979; Jorgenson et al., 1985). A PBPK model for chloroform has been developed by Corley et al. (1990) and applied in a risk assessment context to predict liver tumor incidence by Reitz et al. (1990). The model describes the metabolism of chloroform and the induction of cytotoxicity in the liver after inhalation or oral (gavage or drinking water) exposures.

The most important consideration with respect to the use of PBPK modeling in risk assessment contexts is the definition of dose metrics (also called effective doses or dose surrogates) known or assumed to be related to the endpoint of interest. In the case of chloroform and liver tumors, Reitz et al. (1990) considered two possible dose metrics. The first was a measure of the average daily macromolecular binding in the liver (denoted AVEMMB in the model, with units of $\mu\text{mol/l}$ of liver/day). AVEMMB is a proportion of total daily metabolism (volume and time adjusted) determined by the parameter FMMB as shown here:

$$\text{AVEMMB} = (24/T) * \text{AM} * \text{FMMB} / \text{VL}$$

where T is the duration of the simulation, AM is the total amount of chloroform metabolized, and VL is the volume of liver. The parameter FMMB represents the fraction of metabolized chloroform that binds to macromolecules.

The second dose metric considered by Reitz et al. (1990) was a measure of the percentage of liver cells dying as a result of any exposure; it is denoted PTDEAD and depends on the rate of macromolecular binding ($\text{FMMB} * \text{RAM} / \text{VL}$, where RAM is the rate of chloroform metabolism), the resulting fraction of cells that will eventually die (FRAC), and the rate at which

they die (KDIE):

$$\begin{aligned} \text{PTDEAD} \\ = 100 * \int [\text{KDIE} * \text{FRAC}(\text{RAM} * \text{FMMB} / \text{VL}) \\ - \text{BASAL1}] dt \end{aligned}$$

where the limits of the integration are 0 and T, and BASAL1 is the background rate of cell turnover. PTDEAD depends on $\text{RAM} * \text{FMMB} / \text{VL}$ through the function, FRAC; $\text{FRAC}(x)$ is the probability to the left of x, for a normal distribution with mean MIDPNT and standard deviation SIGMA. Both MIDPNT and SIGMA are parameters of the chloroform PBPK model that require estimation. If one considers the population of liver cells to be normally distributed with respect to their sensitivity to injury (in this case injury associated with macromolecular binding of chloroform metabolites), then $\text{FRAC}(\text{RAM} * \text{FMMB} / \text{VL})$ can be interpreted as the proportion of those normally distributed cells with sensitivity less than $\text{RAM} * \text{FMMB} / \text{VL}$.

The study reported here performed a sensitivity and uncertainty analysis of the Reitz et al. (1990) chloroform PBPK model. In this analysis the term variability is used to denote the variation from one individual to another with respect to the values of the PBPK parameters; the term uncertainty is used to denote the absence of perfect knowledge about the means of the interindividual variability distributions. Specifically, the analysis considered the impacts of parameter variability on the model-predicted values of AVEMMB, PTDEAD, and AM. In addition, the analyses characterized the effect of the variability induced in PTDEAD on liver cancer risk estimates. It was assumed for the sake of the analyses that PTDEAD is the appropriate metric for chloroform risk assessment and interspecies scaling (Reitz et al., 1990), i.e. it was assumed that humans and test species are equally susceptible when they experience exposures entailing the same PTDEAD values. A sensitivity analysis was conducted to determine the parameters to which PTDEAD was more sensitive; uncertainty concerning the mean values of those parameters and

the effect of that uncertainty on risk estimates (added to the uncertainty reflecting variability alone) were also investigated.

It is important to note that the emphasis of this study was on the nature of the approach to be used rather than on the initial result obtained. That is, the purpose of this effort was more to explore the issues and identify the challenges associated with performing a layered variability/uncertainty analysis than to obtain a precise estimate for chloroform. Details concerning the methods of analysis are presented in the next section.

2. Methods

The analyses described below were restricted to chloroform exposure in mice and humans. For the mice, the exposures simulated were those of the 2-year chronic bioassays of the NCI (1976), i.e. 238 and 477 mg/kg/day gavage in females and 60, 138 and 277 mg/kg/day gavage in males (5 days per week). Simulation of those exposures allowed for the estimation of dose metric values (and the associated variability and uncertainty) that could be linked to observed liver cancer incidences for dose-response assessment. The human exposure scenario, for which risk estimates were derived, assumed that oral ingestion of chloroform, at 1 ppm in drinking water, was the only route through which humans were exposed.

2.1. Variability of PBPK model predictions

For each dose group of the mouse bioassays and for the hypothetical human exposure scenario, the variability of AM, AVEMMB, and PTDEAD were determined. The variability was estimated by:

- (1) selecting an appropriate mean value for each of the parameters in the PBPK model;
- (2) for each parameter, specifying a distribution around the mean value, intended to represent only the variability from one individual animal (or human) to another;
- (3) running the PBPK model for each dose group (or human exposure scenario) 500 times, each run having a randomly selected vector of parameter values.

This procedure was done twice for AM, the first time including variability for the partition coefficients and relative tissue blood flow rates, and the second time ignoring that variability. In both cases, the mean values of the parameters required for AM estimation were set as shown in Table 1, and the variability was represented by a normal distribution with coefficients of variation (CV, i.e. the standard deviation divided by the mean) as shown in Table 1. For the relative tissue blood flow parameters and partition coefficients in the first set of runs, the values from Reitz et al. (1990) were used, normal distributions were assumed, and CVs were set as shown below for both mice and humans:

Parameter	Definition	CV ^a
QRC	Relative blood flow to rapidly perfused tissues	0.3
QLC	Relative blood flow to liver	0.15
QSC	Relative blood flow to slowly perfused tissues	0.3
QFC	Relative blood flow to fat tissues	0.3
PB	Blood/air partition coefficient	0.15
PRA	Richly perfused tissue/air partition coefficient	0.15
PLA	Liver/air partition coefficient	0.15
PSA	Slowly perfused tissue/air partition coefficient	0.3
PFA	Fat/air partition coefficient	0.15

^aThe rationale for the selection of CVs will be discussed in the next section, "Selection of coefficients of variation." (Section 2.2).

The mean values for all parameters were taken to be the parameter values reported by Reitz et al. (1990), except for the alveolar ventilation and cardiac output, which were calculated in a manner which considered their observed physiological interrelationship (Astrand and Rodahl, 1970; EPA, 1988). In both sets of runs, and throughout the rest of the analyses described below, cardiac output (with scaling constant QCC) and pulmonary ventilation rate (with scaling constant QPC) were assumed to be bivariate normal random variables with mean values for QCC and QPC of 15 and 30, respectively, in mice, and 15 and 24,

Table 1
Parameters required for AM estimation: Reitz et al. (1990) chloroform PBPK model

Parameter	Definition	Mean		Coefficient of Variation
		Mouse	Human	
BWC	Body weight (kg)	0.0285	70.0	0.15
VSC	Relative volume of slowly perfused tissues	0.74	0.60	0.15
VLC	Relative volume of liver	0.059	0.031	0.15
VRC	Relative volume of richly perfused tissues	0.05	0.05	0.30
VFC	Relative volume of fat tissue	0.06	0.231	0.30
VCC	Relative volume of carcass	0.09	0.09	0.30
VMAXC	Capacity of metabolism (mg/h—1 kg)	22.8	15.7	0.30
KM	Affinity of metabolism (mg/l)	0.35	0.45	0.30
KASTOM	Oral uptake rate constant (/h)	0.6	5.0	0.20
KRESYN	Enzyme resynthesis rate constant (/h)	0.125	0.125	0.30
KLOSS	Enzyme destruction rate constant (/h)	0.000572	0.0	0.30 ^a

^aCoefficient of variation for humans set to 0.

respectively, in humans. The CVs for both QCC and QPC were set to 0.15 and the correlation coefficient was set to 0.75, in both species. This was achieved by defining two independent unit normal random variables, QCCZ and QPCZ, and relating them to QCC and QPC as follows:

$$QCC = QCCP + SIGMAC * QCCZ$$

$$QPC = QPCP + SIGMAP * \{RHO * QCCZ + QPCZ * (1 - RHO^2)^{1/2}\}$$

These equations entail means equal to QCCP and QPCP, respectively, which were fixed at the appropriate values. The standard deviations,

SIGMAC and SIGMAP, were fixed at values consistent with CVs equal to 0.15, and the correlation coefficient, RHO, was fixed at 0.75. As with the CVs for the other physiological parameters discussed below, these values were selected to provide a reasonable correspondence to observations (Astrand and Rodahl, 1970; EPA, 1988) adequate for this preliminary study, but were not directly calculated from experimental data.

Based on the results obtained with AM (see Results, Section 3), the variability of the relative tissue blood flow rates and partition coefficients was ignored in the analyses with AVEMMB and PTDEAD. However, there were additional par-

Table 2
Additional parameters required for AVEMMB and PTDEAD estimation: Reitz et al. (1990) chloroform PBPK model

Parameter	Definition	Mean		Coefficient of Variation
		Mouse	Human	
FMMB	Fraction of metabolism binding to tissue	0.003	0.002	0.3
MIDPNT	Mean cell sensitivity	38	38	0.3
SIGMA	Standard deviation for cell sensitivity	0.227	0.227	0.3
KDIE	Cell-death rate constant	5.92	5.92	0.3
BASAL1	Endogenous cell turnover rate	1.08E-04	1.08E-04	0.3

ameters that were required for estimation of AVEMMB and PTDEAD; the means and variabilities assumed for these parameters are shown in Table 2. Normal distributions were assumed for all of the parameters listed in Table 2.

2.2. Selection of coefficients of variation

Although it is difficult, if not impossible, to clearly separate variability from uncertainty in the absence of specific data, in this analysis we assumed some CVs for representing interindividual variation around the values of the parameters used by Reitz et al. (1990). Estimates of the variabilities of the physiological parameters were based to a large extent on the results of a critical review of the physiological literature (Stan Lindstedt, Northern Arizona University, personal communication) conducted as part of a Physiological Parameters Work Group effort sponsored by the EPA and the ILSI Risk Science Institute (ILSI, 1994). Typical variabilities of partition coefficients and metabolic parameters could be estimated from data for tetrachloroethylene (Gearhart et al., 1993) and methylene chloride (Clewell, 1995). Nevertheless, there are a number of parameters in the model for which little or no data on variability are available. Based on the available data and the rationale discussed below, the CVs selected for representing interindividual variability were uniformly set to either 0.15 or 0.3 (the one exception being the CV for KASTOM).

The rationale for the selection of 0.15 and 0.3 as representative of typical variability was as follows. For a normal random variable, the range from (mean - 3*SD) to (mean + 3*SD) contains almost all (more than 99.7%) of the values that random variable can attain, where SD is the standard deviation. If, for example, we were to believe that interindividual variation could be characterized by most values being between mean/2 and 3*mean/2 (i.e. the variability is restricted mostly to the mean \pm mean/2), then it would be natural to set 3*SD = mean/2. This would lead to a CV = 1/6, or 0.15 rounded off. Similarly, if we were to believe that the interindividual variability must be represented by a

broader distribution, then we could specify that the variability is restricted mostly to the mean \pm mean, which would lead to a CV of 1/3, or 0.3 rounded off. These are the two CVs that were used in this analysis: 0.15 for the parameters for which less variability was assumed and 0.3 for the parameters for which more variability was assumed. For KASTOM, an intermediate value for CV of 0.2 was used. The values selected are for the most part similar to values used in previous studies (Portier and Kaplan, 1989; Bois et al., 1990). Nevertheless, it has been truly said (Hattis, 1990) that "any estimate of the uncertainty of a parameter value will always itself be more uncertain than the estimate of the parameter value."

2.3. Variability of risk estimates

For a chemical such as chloroform, for which the cancer risk is based on animal bioassay results, the estimation of risk depends on relating dose and response on the basis of observations for specific groups of animals (rather than for individual animals). Therefore, the analysis of variability of risk estimates was based on representation of, (a) variability of group-averaged dose metrics, and (b) variability of group response rates.

For each dose group from the mouse bioassays, values of the PBPK model parameters in Tables 1 and 2 (except for the blood flow rates and partition coefficients) were sampled from the distributions indicated in those tables, except that the variances associated with the CVs shown in those tables were divided by the number of animals in the group to obtain the square of the standard error of the mean (SEM²):

$$SEM^2 = \text{Var}/N = (\text{CV} \cdot \text{Mean})^2/N$$

where N is the number of animals in a particular group. The SEM² is the appropriate measures of variability for the average value of a parameter for N animals.

In addition, the number of responders in a dose group was allowed to vary according to a binomial distribution. The statistical parameters of the binomial distribution for each group were N, the number of animals in that group, and p₀, the probability of response estimated for that

group when the multistage model was fit to the actual observations using the dose metrics obtained with the mean values of the PBPK parameters shown in Tables 1 and 2.

The two sampling procedures (of PBPK model parameters to get sets of PTDEAD estimates and of response rates) were repeated 500 times for each sex of mice. The 500 sets of PTDEAD estimates and 500 sets of response rates were randomly matched, generating 500 dose-response data sets. The one-stage version of the multistage model (Crump, 1984) was fit to each dose-response data set. A set of 500 PTDEAD estimates associated with the human drinking water exposure scenario (having the characteristics shown in Table 3) was also generated and randomly matched to the 500 multistage model fits. This last matching resulted in 500 maximum likelihood estimates of risk for the human drinking water scenario of interest. The distribution of these risk estimates provides an estimate of the distribution of risks in a human population as a result of interindividual variability.

2.4. Uncertainty of risk estimates

In addition to the variability represented in the previous two analyses, we were interested in characterizing the impact of uncertainty concerning the true distribution of PBPK model parameters. The parameters for such an investigation were selected by determining the ones to which PTDEAD estimates were more sensitive, i.e. by performing an analytical sensitivity analysis on the Reitz model. That analysis determined the percent change in the model output (PTDEAD estimates) relative to the percent change in the input (a specific PBPK model parameter), the normalized sensitivity. The parameters having normalized sensitivity greater than 0.5 (relative change in the output greater than 50% of the relative change in the input) were candidates for the analysis of uncertainty in risk estimates.

For the selected parameters, distributions for the means were developed so as to represent the uncertainty due to estimation of those means. To incorporate the estimates of uncertainty in the means, in addition to the interindividual variability

around the uncertain mean assumed already, the following two-stage procedure was applied. First, 100 sets of means for the selected sensitive parameters were sampled from the distributions for the means. Second, for each of those 100 sets of means, we generated 500 sets of "average" PBPK model parameters (including PBPK model parameters other than the sensitive parameters), response rates, and PTDEAD values associated with a human 1 ppm drinking water scenario, in exactly the same manner as described in the previous subsection. Thus, for each of the 100 sets of means, we generated the output that would have allowed production of cumulative probability plots for risk. The results of this multi-layer analysis can be summarized by plotting the cumulative frequency curve for variability based on the best estimates of the means, and then superimposing box-and-whisker plots of the distribution of estimates for the 5th and 95th percentiles from the 100 cumulative frequency curves.

3. Results

3.1. Variability of PBPK model predictions

For both sexes of mice and for humans, the inclusion of variability in the blood flow rates and partition coefficients had little or no effect on the output distribution of AM, estimating the total amount of chloroform metabolized. That is, within any given dose group the output variance and the percentiles of AM obtained when blood flow rate and partition coefficient variability was ignored were essentially the same as when that variability was included, suggesting that the variability associated with the blood flow rates and the partition coefficients is a very minor contributor to variability in AM (see also the results of the analytical sensitivity analysis below). Because the proposed dose metrics AVEMMB and PTDEAD (Reitz et al., 1990) depend on the blood flow rates and partition coefficients only through their contribution to AM, the subsequent investigation of variability and uncertainty associated with estimates of those metrics and with risk estimates was com-

Table 3
Summary of distributions of AVEMMB and PTDEAD resulting from variability in PBPK parameters: human exposure^a

	AVEMMB		PTDEAD	
	Run ^b		Run ^b	
	A	B	A	B
Mean	1.4×10^{-2}	1.4×10^{-2}	6.5×10^{-4}	6.8×10^{-4}
Variance	2.8×10^{-5}	2.7×10^{-5}	4.5×10^{-6}	4.4×10^{-6}
Percentiles				
2	5.0×10^{-3}	5.0×10^{-3}	0	0
5	6.3×10^{-3}	6.6×10^{-3}	0	6.2×10^{-23}
10	7.8×10^{-3}	8.1×10^{-3}	4.3×10^{-12}	5.7×10^{-12}
25	1.1×10^{-2}	1.1×10^{-2}	1.2×10^{-7}	9.1×10^{-8}
40	1.3×10^{-2}	1.3×10^{-2}	4.1×10^{-6}	5.2×10^{-6}
60	1.5×10^{-2}	1.5×10^{-2}	7.9×10^{-5}	6.7×10^{-5}
75	1.8×10^{-2}	1.7×10^{-2}	3.9×10^{-4}	3.2×10^{-4}
90	2.1×10^{-2}	2.1×10^{-2}	1.6×10^{-3}	1.6×10^{-3}
95	2.4×10^{-2}	2.5×10^{-2}	2.9×10^{-3}	3.6×10^{-3}
98	2.7×10^{-2}	2.6×10^{-2}	5.9×10^{-3}	7.0×10^{-3}

^aThe human exposure scenario is for consumption of drinking water with 1 ppm chloroform.

^bVariability in blood flow rates and partition coefficients is not considered, but variability in the parameters listed in Table 2 is considered.

pleted without considering variability in blood flow rates and partition coefficients.

The results of the simulations also supported the adequacy of 500 iterations per run; replicate runs yielded remarkably consistent distributions for AM in both mice and humans, as well as for AVEMMB and PTDEAD in humans. In mice, the lower ends of the distributions (the 2nd percentiles) of AVEMMB and PTDEAD varied by several percent from run to run.

As shown in Table 3, when interindividual variability was considered, the range of individual human dose metric values predicted with PTDEAD was extremely broad (from 0 to 7×10^{-3}), while individual dose metrics predicted with AVEMMB only varied over about a factor of five (from 5×10^{-3} to 2.7×10^{-2}).

3.2. Variability of risk estimates

Distributions of risk estimates associated with human consumption of drinking water contaminated with 1 ppm chloroform are displayed in Figs. 1 and 2. The results shown are those calculated on the basis of the female mice, which yielded higher risk estimates than the males. Fig.

1 displays the estimated cumulative distributions of risks based on the AVEMMB and PTDEAD dose metrics. The cumulative distribution for risk based on AVEMMB (circles) displays the classic sigmoid shape for a cumulative distribution function. For risks based on PTDEAD (squares) that shape is not apparent because the dose metric is constrained (clipped) to lie between 0 and 100%

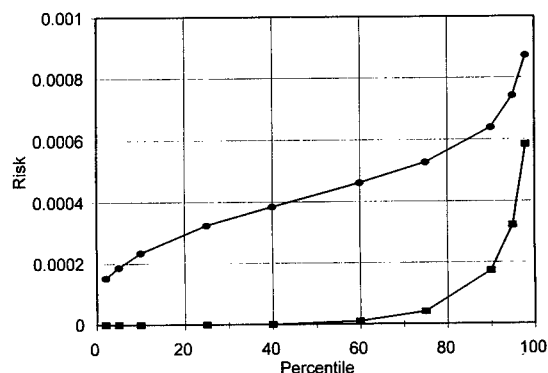


Fig. 1. Distribution of risks in a population for a lifetime exposure to 1 $\mu\text{g/l}$ chloroform in drinking water, based on AVEMMB (circles) and PTDEAD (squares): linear scale.

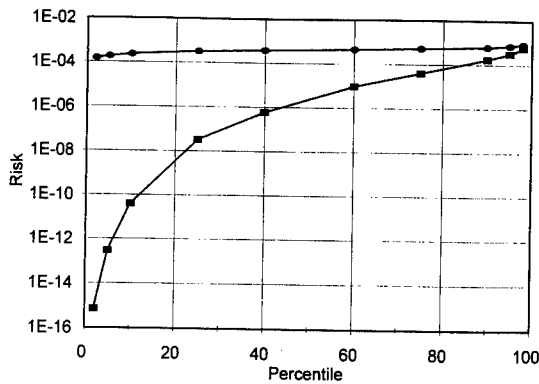


Fig. 2. Distribution of risks in a population for a lifetime exposure to 1 µg/l chloroform in drinking water, based on AVEMMB (circles) and PTDEAD (squares): logarithmic scale.

cell death. The human distribution of PTDEAD was typically zero below the 2nd to 5th percentile (see Table 3), while in several of the animal dose groups, PTDEAD was clipped at 100% above the 90th to 95th percentile. The left tail of the human PTDEAD risk distribution includes extremely small values; the scale of the plot needed

to span the range of PTDEAD values associated with human interindividual variability obscures the lower half of that distribution. Fig. 2 displays the same cumulative distributions as Fig. 1, but using a logarithmic scale for the risk axis so that the lower half of the PTDEAD distribution can be observed.

The range of estimated individual risks across the human population based on AVEMMB is relatively narrow: from 1.9×10^{-4} at the 5th percentile to 7.4×10^{-4} at the 95th percentile, with a mean of 4.4×10^{-4} and a standard deviation of 1.7×10^{-4} . The distribution of predicted human risks based on PTDEAD, however, is extremely broad: from 3.0×10^{-13} at the 5th percentile to 3.2×10^{-4} at the 95th percentile, with a mean of 0.6×10^{-4} and a standard deviation of 1.5×10^{-4} .

3.3. Uncertainty of risk estimates

There is another layer of uncertainty associated with risk estimates that is not represented in the risk distributions presented above. That layer of uncertainty is associated with the fact

Table 4
Normalized parameter sensitivity

Parameter ^a	AVEMMB			PTDEAD		
	Mouse		Human	Mouse		Human
	Lowest dose	Highest dose		Lowest dose	Highest dose	
SIGMA	— ^b	—	—	10.6	3.6	20.6
MIDPNT	—	—	—	-4.2	-4.7	-0.6
FMMB	1.0	1.0	1.0	4.6	4.8	1.3
KDIE	—	—	—	1.0	0.9	1.0
VMAXC	—	—	—	—	3.9	—
KLOSS	—	-0.5	—	—	-0.9	—
KASTOM	—	-0.5	—	3.4	—	—
BWC	—	—	—	—	-1.1	—
VLC	-1.0	-0.7	-1.0	-4.2	-3.8	-0.8
VFC	—	—	—	—	—	0.5

^aSee Tables 1 and 2 for parameter definitions. Parameters not listed had uniformly low (<0.5) normalized sensitivity.

^bLess than 0.5 in absolute value.

that the statistical parameters defining the distributions used to represent interindividual variability of PBPK model parameters are themselves only estimates. They are estimates based on finite samples and hence have quantifiable precision. For the present investigation, this layer of uncertainty was investigated using the subset of PBPK model parameters to which the dose metric PTDEAD was most sensitive.

Table 4 summarizes the results of the analytical sensitivity analysis. PTDEAD appears to be much more sensitive to the parameter values than does AVEMMB. This is true not only for SIGMA, MIDPNT, and KDIE (which affect only PTDEAD and not AVEMMB) but also for FMFB, VMAXC, BWC, and VLC. PTDEAD is sensitive to VMAXC only when the dose is high, i.e. when metabolism of chloroform is reaching saturation. The results of this analytical sensitivity analysis are consistent with the results presented above with respect to the insensitivity of the dose metrics to the blood flow rates and the partition coefficients. Normalized sensitivity greater than 1 (in absolute value) indicates amplification of input error or input variability. Such amplification is a significant cause for concern; therefore the parameters that were considered for the uncertainty analysis were those for which amplification of error was apparent.

The physiological parameters BWC and VLC (body weight and fractional volume of the liver) were considered to be well characterized by their respective distributions representing interindividual variability. That is, because these parameters are easy to measure and can be determined independently of the PBPK model, we assumed that there was little or no uncertainty about the mean values of the interindividual variability distributions. Therefore, uncertainty associated with BWC or VLC was not investigated. On the other hand, the values of the parameter KASTOM (the rate of absorption of chloroform from the stomach) used by Reitz et al. (1990) were based on experience with other, similar compounds. Although uncertainty regarding this parameter is probably significant, there were no data available to address quantitatively the level of uncertainty concerning the mean value,

so KASTOM was excluded from the uncertainty analysis.

The remaining amplifying parameters were VMAXC, FMFB, MIDPNT, SIGMA, and KDIE. The distributions for the means of their interindividual variability distributions were estimated as follows. In all cases, the means were assumed to be normally distributed, because, asymptotically, estimates of means of any distribution should be normally distributed according to the central limit theorem (Bickel and Doksum, 1977).

For VMAXC, it was determined from a comparison of reported values (Corley et al., 1990; Gearhart et al., 1993) that a reasonable range for the mean was 11 to 35 mg/h (scaled to a 1 kg animal). With a best estimate of the mean of VMAXC being 22.8 (Reitz et al., 1990), a normal distribution having variance equal to 37.5 ensures that 95% of the means will be between 11 and 35. That normal distribution was used to characterize the distribution of the mean of VMAXC.

The parameter FMFB represents the ratio between the amount of chloroform bound to macromolecules and the total amount of chloroform metabolized. Expressed in another way, it is the slope parameter for a regression of macromolecular binding on amount metabolized. The variance of an estimate of the slope parameter for linear regression has been well studied in the statistical literature; that variance has been used here to characterize the uncertainty about the value of FMFB. When applied to the same data set used by Reitz et al. (1990) to estimate FMFB, the variance for FMFB was determined to be 5.4×10^{-7} . This variance along with the mean value estimated by Reitz et al. (1990), 0.003, was used to define the normal distribution representing the uncertainty concerning FMFB.

The remaining three key parameters, KDIE, MIDPNT, and SIGMA are related to the cell-killing effect of chloroform in the liver. The estimates of these three parameters are correlated with one another because they were simultaneously estimated from the same set of data; the data available were not sufficient to provide independent estimates of those parameters. The char-

acterization of the uncertainty concerning those parameter values took into account the correlation by considering the uncertainty distributions to be correlated trivariate normals. The means of those distributions were chosen to be those estimated by Reitz et al. (1990): KDIE = 5.92, MIDPNT = 38, and SIGMA = 0.227. The variance-covariance matrix for these parameters was estimated by running the PBPK model using those means and comparing the PTDEAD estimates to the cell killing data used by Reitz et al. (1990, see their Table 5). The software package SCoPFit (National Biomedical Simulation Resource, Duke University) allowed estimation of the variance-covariance matrix when the predictions of the model were compared to the set of reference data; the results of the SCoPFit run were as follows:

$$\begin{aligned}\text{VAR}(\text{KDIE}) &= 2.85 \times 10^3 \\ \text{VAR}(\text{MIDPNT}) &= 3.27 \times 10^3 \\ \text{VAR}(\text{SIGMA}) &= 2.70 \times 10^{-3} \\ \text{COV}(\text{KDIE}, \text{MIDPNT}) &= 3.05 \times 10^3 \\ \text{COV}(\text{KDIE}, \text{SIGMA}) &= -2.56 \\ \text{COV}(\text{MIDPNT}, \text{SIGMA}) &= -2.68.\end{aligned}$$

The values shown above were used for the representation of the uncertainty concerning KDIE, MIDPNT, and SIGMA. Unfortunately, when mean values for these parameters were selected randomly from the distributions described above, a sizeable proportion of the KDIE and MIDPNT simulated means were less than zero. Because negative values of those parameters are not biologically possible, simulations with negative KDIE or MIDPNT mean values were thrown out and replaced by other randomly selected sets of values that had KDIE and MIDPNT greater than zero. However, this resampling process produced an undesired change in the distributions of the parameters: the means of the distributions were shifted to the right (to higher values), reflecting the truncation of the distribution on the left (at zero). As a result, the predicted distributions for the dose metrics were also shifted; for example, the median of the 95th percentiles for the 100 distributions (0.014) was substantially greater than the estimated 95th percentile obtained from the best estimates of the

means (2.7×10^{-4}). Therefore, the results of the uncertainty analysis could not be considered accurate, and are not presented.

4. Discussion

The important features of the uncertainty analysis approach presented here are that: (1) unlike other analyses (Bogen and Spear, 1987; Portier and Kaplan, 1989; Bois et al., 1990), an attempt has been made to differentiate between the impacts of interindividual variability and parameter uncertainty; and (2) an attempt has been made to account for correlation between key parameters.

4.1. Variability of PBPK model predictions

From a biological plausibility viewpoint, it should be a matter of concern that for four of the five animal doses, the distribution for the PTDEAD dose metric reached the maximum value (100% of the cells in the liver dying every day) at the 90th or 95th percentile. That is, for a small but significant portion of those distributions, PTDEAD had reached its maximum value. This occurrence also impacts the risk estimate distribution, since the risk depends on the ratio of the dose metric in human and mouse. However, the more important point is that it is highly unlikely that an animal could survive a chronic exposure in which a large fraction of the cells in its liver were being killed every day, so the upper ranges of all of the PTDEAD distributions in the mice are implausible.

4.2. Variability of risk estimates

The results of this risk distribution analysis can be compared with the risk estimates calculated by Reitz et al. (1990). In that paper, the 95% lower confidence limits on the dose associated with 1×10^{-5} risk from chloroform in drinking water were reported to be 10.8 mg/l, based on female mice, and using the PTDEAD dose metric. This lower confidence limit on dose for a specific risk can be converted to an upper confidence limit on risk for a specific dose. When this conversion is performed, the value reported by Reitz et al. (1990) corresponds to a 95% upper

bound on risk of approximately 9.2×10^{-7} for a dose of 1 mg/l. This risk estimate was also verified using the linearized multistage model and the chloroform PBPK model predictions for PTDEAD based on the parameter values in Reitz et al. (1990). This risk is a factor of 65 below the mean of the risk distribution in this study (6.0×10^{-5}) and more than 300-fold below the 95th percentile of the risk distribution (3.2×10^{-4}). A similar comparison for AVEMMB produced much better agreement: 5.3×10^{-4} based on the preferred values, as compared to the mean and 95th percentile of the risk distribution at 4.4×10^{-4} and 7.4×10^{-4} , respectively. Unfortunately, as pointed out by Reitz et al. (1990), the AVEMMB dose metric does not provide a good correspondence with the tumor incidence in the animal bioassays, while the PTDEAD dose metric does.

The difference between the risk estimates reported by Reitz et al. (1990), and the corresponding values in this analysis provides a measure of the impact of model parameter variability on the distribution of risk estimates. The wide disparity in the case of risks based on cytotoxicity reflects the fact that the use of a normal distribution of cell responses in the calculation of the PTDEAD dose metric in the Reitz et al. (1990) chloroform model is a highly non-linear construct, greatly amplifying the variation in several of the input parameters. As a result, the distribution of risks based on PTDEAD that is produced by varying the chloroform model parameters is very broad. In a similar analysis with a PBPK model for methylene chloride (Clewell, 1993, 1995) the 95th percentile of the risk distributions produced by varying the pharmacokinetic parameters were only a factor of four greater than the corresponding risks predicted with the preferred parameter values. In the case of chloroform risks predicted on the basis of the cell death dose metric, PTDEAD, this ratio is nearly 400. This considerable difference is consistent, however, with the difference in the sensitivity of the two models to their parameters. None of the parameter sensitivities in the methylene chloride model were greater than one (Clewell et al., 1994). In contrast, several of the key parameters in the chloro-

form model evaluated in this study possessed sensitivities for PTDEAD that were between 4 and 20 in magnitude (Table 4).

In summary, although the best estimates of the average population risk (Reitz et al., 1990), based on the preferred dose metric, PTDEAD, are nearly three orders of magnitude lower than those based on AVEMMB (on the order of 10^{-7} vs. 10^{-4}), the impact of parameter variability on PTDEAD is so great that the 95th percentile of individual risks considering pharmacokinetic variability were very similar (in the order of 10^{-4} using either dose metric).

4.3. Uncertainty of risk estimates

The analyses reported here were not successful in representing the contribution of uncertainty about the means of the distributions for the key parameters, i.e. VMAXC, FMMB, KDIE, MIDPNT, and SIGMA. As noted above, the treatment of this uncertainty resulted in a shift in the distributions of the 5th and 95th percentile relative to the values of those percentiles obtained when uncertainty about the means of those parameters was ignored. If the treatment of those uncertainties had been successful, the median of the 5th and 95th percentiles would have been close to the value obtained when the uncertainties were ignored.

The shift in the percentile distributions was a direct result of the fact that the distributions obtained for the means of KDIE and MIDPNT (normals with means of 5.92 and 38, respectively, and standard deviations of 53.3 and 57.2, respectively) allowed negative values for those means. In an attempt to overcome this biologically implausible outcome, the negative values were replaced by resampling from the distribution of the means, but this resulted in median values of the means that were quite different from (greater than) the values 5.92 and 38. Moreover, the problem was exacerbated by the fact that SIGMA was negatively correlated with both KDIE and MIDPNT. When larger, positive values of those two parameters replaced negative values, the randomly selected replacement SIGMA values tended to be less than those they were replacing.

The effect of changes in these sensitive parameters on the predictions for PTDEAD in the mouse and human, and the resulting impact on the human risk estimate, can be understood by referring to the sensitivities shown in Table 4. For example when KDIE increases, both the mouse and human PTDEAD dose metric are increased to a similar extent (because the sensitivities are similar). Therefore, the human risk estimate, which depends on the ratio of the dose metrics in the human to those in the mouse, is not likely to be strongly effected. However, when MIDPNT increases, the magnitude of the sensitivity of PTDEAD in the mouse (4.2-4.7) is much greater than in the human (0.6). Since all of the sensitivity coefficients for MIDPNT are negative, an increase in MIDPNT always produces a decrease in PTDEAD. The greater extent of the decrease in PTDEAD in the mouse (due to its greater sensitivity to MIDPNT) relative to the human leads to an increase in the ratio of the human dose metric to that of the mouse, and thus to an increase in the risk estimate. On the other hand, when SIGMA increases, the sensitivity of PTDEAD in the mouse (3.6-10.6) is less than in the human (20.6), and the positive sign of the sensitivity coefficients for SIGMA indicates that an increase in SIGMA produces an increase in the dose metric. The greater extent of the increase in PTDEAD in the human than in the mouse leads again to an increase in the ratio of the human and mouse dose metrics and thus to an increase in the risk estimate.

The generation of negative values for the cell response parameters was the result of the large variance calculated for the distributions of the cellular response parameters and the fact that the normal distribution is unbounded on the left. It might have been possible to overcome the difficulty associated with negative values by the use of lognormal distributions, which cannot produce negative values. However, the lognormal distribution is highly skewed, with a significant tail on the right, raising the concern that the resulting risk distributions would still be artifactually altered, but in a more subtle way, misrepresenting the actual effects of parameter uncertainty. There is no basis for suggesting that

the cellular response parameters are lognormally distributed, in fact it is unlikely that they are.

Despite the problems with the treatment of uncertainty about interindividual variability distributions, it is possible to draw some conclusions about the effect of that uncertainty. Clearly, the data set used to estimate the key parameters KDIE, MIDPNT, and SIGMA fails to provide very precise estimates of those parameters. This is a crucial factor with respect to uncertainty of risk estimates as long as the PTDEAD dose metric is assumed to be the measure appropriate for animal-to-human extrapolation. PTDEAD is exquisitely sensitive to KDIE, MIDPNT, and SIGMA. Unless and until more data are available for estimating these key parameters, uncertainty concerning the effect of chloroform macromolecular binding on liver cytotoxicity will limit our ability to get precise risk estimates. Moreover, the results of the short-term experiments used to estimate the relationship between chloroform exposure and cytotoxicity in the model of Reitz et al. (1990) do not reflect the significant changes in tissue response that occur as a result of chronic exposure to chloroform (Larson et al., 1993, 1994). Additional data on the cell proliferation associated with chloroform exposure are now available (Larson et al., 1993, 1994); these new data have been obtained from subchronic exposures and provide a much better representation of the bioassay conditions.

4.4. Other uncertainties

This investigation illustrates the considerations and procedures that are necessary to characterize the impact of interindividual variability and pharmacokinetic parameter uncertainty on risks estimated with a pharmacokinetic model. However, the variability and uncertainty addressed by the methodology presented here relate only to the parameters of the PBPK model. These are uncertainties that are more amenable to quantitative treatment and therefore have been explored in greater detail, here as well as elsewhere (Bogen and Spear, 1987; Farrar et al., 1989; Portier and Kaplan, 1989; Bois et al., 1990; Clewell, 1993, 1995). The other uncertainties that

are more difficult to treat quantitatively, but which could have an important impact on risk estimates, relate to the structure of the PBPK model, the choice of dose metric, and pharmacodynamic differences among species, among others. For example, the Reitz et al. (1990) model assumes a particular structure for describing liver cell sensitivities to chloroform macromolecular binding, namely a normal distribution of sensitivities. This particular structure is a construct and other constructs are possible; until appropriate experiments are conducted to support or refute this representation, uncertainty will be associated with its use. An alternative model of chloroform has been described (Beck et al., 1993) that uses an empirical link between the average rate of metabolism over the recent time period and the number of cells dying, rather than imputing a normal distribution to this relationship.

4.5. Possible refinements to approach

In several respects, the results of the analysis reported here are only preliminary. As mentioned earlier, the purpose of this effort was more to explore the issues and identify the challenges associated with performing a layered variability/uncertainty analysis than to obtain a precise estimate for chloroform. First, and most importantly, most of the values selected for the coefficients of variation and correlation for the model parameters were only rough approximations based on limited data and experience, as can readily be seen from the rationale provided in the methods section. The analysis would clearly be improved by a more careful evaluation of actual experimental data on the variation of the parameters used in the model, particularly those to which the model is more sensitive.

Secondly, for the most part the sensitivity and uncertainty analyses performed in this study were univariate; that is, potential correlations among most of the parameters were ignored. It should be noted, however, that one of the novel features of the approach used in this study was that the correlations among what appear to be the most important model parameters were indeed considered, and an attempt was made to deal with them. Nevertheless, the more correct way to

perform the analyses would have been to use a fully multivariate analytical approach throughout.

Finally, the critical factors producing the broad uncertainty of risk estimates from the chloroform model are the sensitivity of the model to the parameters defining the normal distribution of susceptibilities and the limited data available for their identification. The additional data now available on the cell proliferation associated with chloroform exposure (Larson et al., 1993, 1994) could be analyzed in a manner similar to that used by Reitz et al. (1990) to revise the parameter estimates for the model. The impact of the new parameterization on the predicted risks and their associated uncertainty could then be evaluated as described in this study. In addition, the alternative model of chloroform (Beck et al., 1993) specifically designed to make use of this new data could also be evaluated. A comparative evaluation of the two modeling approaches would provide information on the extent to which the new data and model structure reduce the uncertainty associated with the incorporation of cytotoxicity data into a risk assessment for chloroform.

5. Conclusion

The present investigation has suggested an approach that is appropriate for the separate, quantitative treatment of variability and uncertainty associated with risk assessments based on a PBPK model. To incorporate estimates of uncertainty in the means, in addition to the interindividual variability around the uncertain means, a two-stage procedure was described. First, 100 sets of means for selected sensitive parameters were sampled from the uncertainty distributions for the means in both the animal and the human. Second, for each of those 100 sets of means in both species, 500 sets of the PBPK model parameters (including the less sensitive parameters) were sampled from their respective variability distributions. Animal and human dose metrics were then predicted with the PBPK model using each of these 100 sets of 500 parameter vectors, and the risks for each of the

50 000 cases were estimated using the multistage model and resampled response rates. Thus, if desired, cumulative probability plots for risk could be generated for each of the 100 sets of means. However, the results of this multi-layer analysis can be summarized by plotting the cumulative frequency curve for variability based on the best estimates of the means, and then superimposing box-and-whisker plots of the distribution of estimates for the 5th and 95th percentiles from the 100 cumulative frequency curves.

An important feature of the analytical approach was that an attempt was made to incorporate information on correlation between important parameters, for example the observed correlation between total blood flow and alveolar ventilation rate. The difficulties encountered in this study of chloroform resulted from an unusual degree of sensitivity of the PBPK model predictions to highly uncertain parameters. Therefore, application of this approach to PBPK risk assessments for other chemicals where parameter uncertainty is less pronounced would still seem to be appropriate and useful. The value of the approach described in this investigation is that it would allow the risk assessor and risk manager to simultaneously visualize the dispersion of PBPK risk estimates induced by interindividual variability and model parameter uncertainty.

Acknowledgements

This study was supported by the USEPA Office of Drinking Water. However, the views presented in this paper are strictly those of the authors and do not necessarily reflect the position of the agency. The authors would like to thank Tim Barry of the USEPA Office of Drinking Water, who recognised the need for this study and helped to define the overall goals of the effort.

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ELSEVIER

Toxicology 111 (1996) 305-313

TOXICOLOGY

Risk assessment policy for evaluating reproductive system toxicants and the impact of responses on sensitive populations

G. Bruce Briggs

*Geo-Centers, Inc., Naval Medical Research Institute Detachment (Toxicology), Building 433,
Area B, WPAFB, OH 45433-7903, USA*

Abstract

Risk assessment policy for evaluating environmental chemicals for their potential to produce reproductive system failures is similar to policy for evaluating cancer-causing effects. The objective of reproductive system risk assessment is to expand on the test standards that primarily focus on fertility endpoints and birth defects by using mechanism-of-action studies and quantitative risk assessment methods. An understanding of the sensitivity of reproductive system insult between animal species and from animal models to man is critical to developing risk assessment policy and test standards. The reproductive process is complex and involves a number of maturation and sex cell development processes. Sensitivity to insult varies throughout this process, especially during, (1) the development of the conceptus, sperm and ova, (2) fertilization, (3) implantation, and (4) puberty. Reproductive failure has many causes and clinical effects. Risk assessment policy is directed toward reducing the uncertainty associated with the cause by providing a guide to understanding how dose, duration, and characteristics of the reproductive toxicant affect the reproductive process.

Keywords: Developmental toxicology; EPA risk assessment policy; Extrapolating toxicity data; Reproductive toxicology; Reproductive system risk assessment; Sperm staging

1. Introduction

Claude Bernard established the basic principles for toxicologic evaluation more than 100 years ago. They remain as valid today as when they were first written and are the foundation for safety evaluation research and risk assessment. This presentation will examine several of those principles and relate them to the policies that are shaping the current regulations which guide the risk assessment of potential reproductive toxicants.

Three of these principles are key factors in risk assessment policy development:

- (1) Physiologists must discover the laws of "vital manifestations" or physiological functions, and observation and examination are the only methods of investigation.
- (2) Toxicity to target organs is determined by establishing approaches to defining the mechanism of action of drugs and other chemicals.
- (3) Cause and effect relationships are established through developing an objective and a hypothesis, conducting the examination and controlling the variables.

This review presents the major reproductive risk assessment policies that are currently being used. It also discusses the "new generation" methodologies to improve the information relating to potential risk to sensitive populations; the gametes, the conceptus and the adult male and female.

2. Reproductive system risk assessment policy development

The science relating to the toxic insult of the reproductive system has been driven by the need to prevent exposures from reproductive and developmental toxicants to sexually active adults. Policies have evolved primarily through a series of guidelines and regulations that rely on laboratory animal surrogate models and standardized test standards that have been developed to link the causes and clinical effects of reproductive system failures (see references in Section I). The Food and Drug Administration (FDA) issued the original test standards in 1966. These guidelines established the requirements for regulatory approval of new drugs under development. Segment I Reproductive Effects Studies were proposed to evaluate fertility in rats. Segment II Teratology Studies were proposed to be conducted in a rodent and non-rodent animal model to evaluate birth defects and malformations in the offspring. Segment III Perinatal and Postnatal Studies were developed to evaluate potential toxicity to the young during lactation and early development. Multigeneration reproduction studies were also recommended for some pesticides that could enter the food chain of humans. A battery of neurobehavioral tests was established at a later date to evaluate potential developmental effects on the sensory organs and the central nervous system during pregnancy.

These tests have served the regulatory process and society by using animal surrogates as test models to avoid catastrophic toxic insult to the human reproductive system. They have not been revised since they were proposed and focus primarily on fertility endpoints of malformations, functional defects, growth retardation or death.

One approach for setting acceptable levels for developmental toxicity risk has been to use safety (uncertainty) factors. From a bioassay conducted at several dose levels in both a rodent and a non-rodent animal species, a supposedly safe dose for humans is determined by dividing the no-observable-adverse-effect level (NOAEL) by a safety factor. It has been suggested that a safety factor of 100 should be used when extrapolating from animal study data to establish acceptable human exposure levels (Lehman and Fitzhugh, 1954, Section VI). If the NOAEL is taken to be a safe dose for the experimental animals, a safety factor of 10 is suggested to allow for potentially higher sensitivities of humans compared to the experimental animals and another factor of 10 to allow for differences in sensitivities among individuals. For irreversible effects, such as death or malformation, an additional safety factor of 10 is suggested (Jackson, 1980, Section VI). Even though the safety factor of 100 is adequate to account for interspecies and intraspecies differences in response, this does not necessarily result in a risk-free dose because the power of the experiment may not be adequate to detect subtle toxic effects (Galor, 1989, Section VI). Although this method is not foolproof for setting acceptable levels for humans, it has provided a margin of safety and has reduced the risk for most chemical entities that have been evaluated by using these standardized tests. Most teratological studies are capable of detecting reproductive system disease incidence of 10% or more. The reason for this increase in sensitivity in humans to reproductive system toxicants is not clearly understood but is likely due to differences in metabolism and mechanism of action of the hazardous chemical. Warning labels are required for drugs and pesticides to alert physicians and sexually active humans to avoid contact with developmental toxicants, especially during pregnancy. The primary regulatory concern today is that data gaps exist for most of the chemicals in commerce, and more than 4000 reproductive or developmental toxicants for animals do not produce these effects in humans. About 50 human reproductive system toxicants have been reported to have caused developmental toxicity in humans (Schwetz and

Harris, 1993, Section I). The regulatory policy has been largely based on preventing or reducing exposure to the mother at or below safe levels during the sensitive periods for fetal development. Prevention of exposure to human toxicants remains to be the most effective principle for protecting the reproductive system from toxic insult (see references in Section IV).

Since the promulgation of the FDA guidelines, a number of position papers, guidelines or regulations have been written to establish policy for protecting humans from reproductive system risks. Several of these policies are discussed in this presentation and a list of policies is included.

3. Discussion

New risk assessment policy for going beyond the current test battery to explore the processes by which reproductive system failures and successes occur has been promulgated (see references in Section II). This policy, along with the development of new models for expanded end points that characterize mechanisms of sex cell maturation and function, is providing scientists and regulators better means for assessing chemical risks. The U.S. Environmental Protection Agency (EPA) has developed risk assessment guidelines which were finalized in 1991 (see references in Section I, 1991). These guidelines were based on the same criteria for cancer risk assessment which are routinely used today. These include:

- (1) Hazard identification
- (2) Dose-response assessment
- (3) Exposure assessment
- (4) Risk characterization

These guidelines define reproductive toxicity and developmental toxicity and describe and discuss the endpoints that need to be evaluated in order to prevent adverse effects to the reproductive system and process. The EPA guidelines define reproductive toxicity as the occurrence of adverse effects on the reproductive system that may result from exposure to environmental

agents. Toxicity may be expressed as alterations to the reproductive organs and/or the related endocrine system. The manifestation of such toxicity may include, but not be limited to, alterations in sexual behavior, onset of puberty, fertility, gestation, parturition, lactation, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive system. Developmental toxicity is defined by the EPA as the occurrence of adverse effects on the developing organism that may result from exposure before conception, during prenatal development, or postnatally to the time of sexual maturation. Adverse developmental effects may be detected at any point in the life span of the organism. The major manifestations of developmental toxicity include death of the developing organism, structural abnormality, altered growth, and functional deficiency.

Fertility and reproductive function in both males and females are evaluated in the laboratory rat in the Segment I study. The reproductive toxicity is evaluated by dosing both sexes with at least three dose levels. Sexually mature rats are dosed through a sperm cycle and female sexually mature rats are dosed with three dose levels of the test article for 14 days prior to mating. For most chemicals, only one generation is required. The offspring are then evaluated for individual and litter effects of toxicity. The U.S. Food and Drug Administration uses multigeneration studies for food additives to evaluate chemical effects on fertility, gestation, parturition, lactation development and offspring development and reproduction. Segment II developmental toxicology studies are conducted in a rodent and a non-rodent species. Pregnant rats are exposed during the period of organogenesis during days 6-15 of gestation, and pregnant rabbits are exposed on days 6-18. The pregnant dams are terminated one day prior to delivery, and the pups are examined for viability, malformation, and growth (Manson and Kang, 1994, Section VI C). The International Harmonisation Committee Guideline, (ICH, 1994, Section I), recommends the supplementation of the standardized tests with staging techniques and mechanism of action

studies. Many of these new methods are described in the references by Heindel and Chapin. (Heindel and Chapin, 1993; Chapin and Heindel, 1993, Section VI C).

The risk assessment policy guidelines have improved the earlier test standards by stating the assumptions made in the risk assessment process and standardizing the use of qualitative and quantitative data in the hazard identification and dose-response processes. The guidelines have also helped to identify research needed for reducing uncertainties and to fill data gaps. This information is included in databases (see references in Section IV), and is used along with epidemiology facts (see references in Section III), to perform a reproductive risk assessment for potential reproductive system toxicants.

The criteria that are used for cancer risk assessment are used for the risk assessment of reproductive and developmental toxicants with the following additional assumptions:

- (1) An agent that produces an adverse reproductive effect in experimental animals will potentially pose a hazard to humans after sufficient exposure.
- (2) Reproductive effects are generally the same across species except for pregnancy outcomes.
- (3) All of the manifestations of developmental toxicity are of concern, including growth alterations, functional deficits and fetal death, in addition to structural abnormalities.
- (4) A threshold is generally assumed for the dose-response curve for reproductive effects.

Standardization of data collection has been the primary objective of the International Harmonisation Committee (IHC) guidelines that were published in 1994 (IHC, 1994, Section I). This document introduces the concept of "most probable option" which is interpreted as optimizing the test parameters to reflect sound scientific procedures. This includes determining the optimal treatment period for both male and female animal models and the conceptus so that exposure to the toxicant occurs during the most sensitive period of maturation and development. The testing requirements will include general screens to identify potential treatment-related effects and studies to characterize the nature, scope

and/or origin of the toxic effect. The screening studies will remain essentially the same as with previous guidelines. The characterization studies include optimization of test designs for kinetic and metabolism studies in pregnant/lactating animals and male fertility assessment.

The IHC recommendations for male fertility assessment includes the requirement for dosing animals prior to mating for at least one sperm maturation cycle, and performing sperm evaluation studies in addition to histological evaluation. This is accomplished by incorporating methods to evaluate sperm motility and morphology using computer assisted techniques and the staging of spermatogenesis (Russell et al., 1990, Section VI C). This process can also be used for oogenesis. The ovarian follicles are all present when the female is born. They exist as primordial follicles until they are individually stimulated by hormones to develop into primary follicles, secondary follicles, early tertiary follicles and Graffian follicles. The oogenesis process involves distinct mitosis and meiosis stages. Each of these stages can be identified by characteristic structures that demonstrate the maturation and differentiation of the ova. The ability to determine the stage that the toxic insult occurred is a primary consideration in understanding how the reproductive failure was produced. The first sensitive stage of the maturation process of the gamete dictates the expression of the reproductive failure. The reproductive process is a continuum of cell growth and function. The usual end point of a toxic insult early in the development of the gamete is death to the gamete. Since there are many millions of sperm being produced simultaneously, there must be a massive insult to a majority of the sperm. This insult is detected by a loss of motility and normal morphology. Human reproductive failure can result from only a slight reduction in the number of viable sperm, but the rat has been shown to be able to produce offspring with viable sperm counts of approximately 20% of normal. Ova can be insulted at any stage of maturation, but the most rapid development stages of meiosis when the Graffian follicle is becoming functional is usually the most sensitive period for toxic insult. The "trigger points" for cause and effect relationships can occur at any

point along the maturation process. The toxic insult to the sperm can produce infertility by interfering with the locomotion process or by causing biochemical changes that interfere with fertilization. The ova may lose viability and cause infertility and early reproductive failure in animal models and premature menopause in women.

Once the stage and cell types that are affected are identified, biomarkers can be used to help develop an understanding of the mechanism of action that produced the adverse event. These biochemical markers include hormones, enzymes, DNA adducts, biochemical substrates and metabolic pathway (cytochrome P-450) pathway changes. These changes can be detected in the plasma, or the tissues of the reproductive system (see references in Section IX). This combination of assessment of the gamete maturation process, mating behavior, fertility, pre-implantation stages of the embryo, and implantation provide data for the most probable option risk assessment.

New methods models and processes have been developed to help understand the mechanism of action in the normal and abnormal reproductive process in sensitive populations (see references in Section VIII). A number of new biomarkers are currently being used to define the reproductive process and detect alterations in function that result in reproductive failures (see references in Section V). These are driving policy development and are providing a body of information that is providing the associations between causes and effects in reproductive failure. By examining similarities and common sensitivity patterns between animal species and from animals to man, an optimum risk assessment approach can be developed for environmental toxicants that affect reproduction.

New methods for utilizing all the data from animal studies for extrapolating to man (see references in Section VI A), and incorporating statistical methods (see references in Section IX A), have contributed to our ability to assess reproductive system risk. Benchmarking, comparisons of fetal-to-adult effects (A/D Ratio), and improvements in the reference dose calculations and categorical regression procedures are all being considered for improving the value of the animal data for scaling to humans (see references

in Section VI B). Standardization of test standards and comparisons of similar endpoints are critical for making risk assessments from study to study and from chemical to chemical.

Pharmacokinetic and pharmacodynamic principles can be applied for gestational and lactational modeling using physiologically-based pharmacokinetic (PBPK) procedures (see references in Section IX B).

The ultimate goal of reproductive system risk assessment policy is to provide a practical and affordable method for reducing the risk from toxicants to an acceptable level (see references in Section VII). The challenge for regulators and scientists is to reduce the variables in examining potential risk factors and to link cause and effect parameters to reflect real world scenarios.

4. Conclusion

This presentation has briefly reviewed the process of reproductive risk assessment policy development and how it relates to methods and test standards that are being used to generate and evaluate the data for regulatory decision making. The conceptus and the adult male and female that are attempting to produce offspring are the most susceptible human populations to reproductive system toxicants. The reduction in variables improves the process of extrapolating data from experimental animals to humans. Reducing exposure levels to an environmental chemical or group of chemicals during sensitive periods across the human population has been the objective of regulatory agency policies, especially since 1983. Dose, duration and characterization of the risk factor(s) combined with the time of the insult and mechanism of action for reproductive failure are the primary factors in developing policies that guide reproductive system risk assessments (see references in Section VII).

Acknowledgements

The author is indebted to Mrs. Lucie Connolly for reviewing and typing the references, and to Ms. Lana Martin for technical editing of the text. This presentation was supported by the Navy

Environmental Health Center Work Unit No. 1323. The opinions and assertions herein are private to the author and are not to be construed as official or as reflecting the views of the U.S. Navy Department or the Navy Service at large.

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ELSEVIER

Toxicology 111 (1996) 315-329

TOXICOLOGY

Use of physiologically based pharmacokinetic modeling to investigate individual versus population risk

Harvey J. Clewell III*, Melvin E. Andersen

K.S. Crump Group, ICF Kaiser International, 602 E. Georgia Avenue, Ruston, LA 71270, USA

Abstract

Because of the heterogeneity of the human population, it is generally expected that there will be a broad range of observed susceptibilities to the biological effects of exposure to chemicals or drugs. Often it is possible to distinguish specific classes of individuals, such as infants or the elderly, who appear to be more susceptible to a specific effect. Non-cancer risk assessments often address this variability by dividing the experimentally determined acceptable exposure level by an uncertainty factor of 10 to protect sensitive individuals; cancer risk assessments typically do not address this issue in any quantitative fashion. Physiologically based pharmacokinetic (PBPK) modeling provides the capability to quantitatively describe the potential impact of pharmacokinetic factors on the variability of individual risk. In particular, PBPK models can be used to determine the impact of differences in key metabolism enzymes, whether due to multiple genotypic expression, such as cytochrome P450 polymorphisms, or just due to normal variation in enzyme activities within the general population. Other potential modulators of sensitivity which can be addressed quantitatively with a PBPK model include physical condition, level of activity, disease states, age, hormonal status, and interactions with other chemicals and drugs. In each case, the PBPK model provides a quantitative structure for determining the effect of these various factors on the relationship between the external (environmental) exposure and the internal (biologically effective) target tissue exposure. When coupled with Monte Carlo analysis, the PBPK model provides a method to assess the quantitative impact of these sources of variability on individual risk (as opposed to average population risk) by comparing model-predicted risks over the distribution of individual parameter values.

Keywords: Pharmacokinetic modeling; Risk assessment; Variability; Sensitive subpopulations

1. Introduction

It is a common observation that individual people do not respond in the same way and to the same extent to exposures to toxic chemicals. For instance, studies of workers exposed to vinyl chloride have repeatedly shown an increased in-

cidence of a rare form of liver cancer (Jones et al., 1988; Simonato et al., 1991). Although an increased incidence is observed even in the lower exposure categories in these studies, most of the workers in the highest exposure group do not get cancer. This disparity results in part from the fact that cancer is a stochastic process, but it is likely that it also reflects the growing evidence that some individuals are more susceptible than

*Corresponding author.

others to certain forms of cancer. As another example, in a study of an inadvertent short-term exposure of families to high concentrations of manganese in their drinking water (Kawamura et al., 1941), it was noted that the greater the ages of the exposed individuals, the more severe were the symptoms. In fact, none of the children of ages 1-10 showed any signs of intoxication, while severe symptoms, including death, were observed in the much older individuals.

2. Sources of interindividual variability

2.1. Exposure

The risk, if any, that a person will incur toxicity as a result of chemical exposure, as well as the magnitude of any injury, is of course determined in part by the extent of the exposure. One of the important trends in exposure assessment is the growing use of Monte Carlo techniques, in which a distribution of potential exposures is used in place of a single "worst case" estimate to obtain the anticipated distribution of risks in a population (Hattis and Burmaster, 1994). Briefly, in the Monte Carlo method a probability distribution for each of the input parameters (concentrations in various media and exposure pathway factors) is randomly sampled, and the exposure assessment is run using the chosen set of parameter values. This process is repeated a large number of times until a probability distribution for the population has been created. To the extent that the input parameter distributions adequately characterize the variability in the inputs, and assuming that the parameters are reasonably independent, the resulting output distribution will provide a useful estimate of the distribution of exposures in the population.

It is important to note that a proper exposure estimate must be based on the complete pattern of exposure, including both the intensity and duration, rather than just an average measure; the delivery of a chemical to the site of toxicity and the generation of the toxic effect are often complex functions of the external exposure (Hattis and Burmaster, 1994). Vinyl chloride is an example of a chemical for which metabolism is the limiting factor in the production of toxicity.

At low concentrations, the incidence of tumors is clearly dose-related, but at exposures above saturation of metabolism (around 250 ppm) little further increase in tumor incidence is observed (Gehring et al., 1978). Thus, exposure to 100 ppm for 8 h per day would be much more potent than exposure to 800 ppm for 1 h per day. Manganese, on the other hand is an example of an essential nutrient which has also been found to be toxic at high exposure levels (Schroeder et al., 1966). In this case, chronic environmental exposures within the range of normal dietary intakes are unlikely to be harmful because the element is under homeostatic control; however, exposures sufficiently high to disrupt homeostasis, even for a short period of time, can lead to toxicity.

2.2. Response

In addition to interindividual differences in exposure, there can also be large differences in individual responses to a given exposure. The variability of individual responses to a chemical exposure is well known even in testing of highly inbred animal species, in which interindividual variability is greatly reduced compared to an outbred species such as humans. This variability of response is generally described by a probit analysis in which the log of the exposure concentration or dose is plotted against the proportion of the population responding; the familiar LD_{50} and LC_{50} represent a central measure for the observed distribution of responses. The point here is that, under identical exposure conditions, some individuals will evidence a response to a particular chemical exposure and others will not. A large number of factors come into play in determining the likelihood that a particular individual will display a toxic response from a given exposure to a chemical. These can be divided into two main categories: hereditary factors and environmental factors.

2.2.1. Hereditary factors

The different susceptibility of individuals to cancer has often been associated with a genetic predisposition, e.g. retinoblastoma (Moolgavkar and Knudsen, 1981). Genetic differences in the enzymes responsible for activating or detoxifying

Table 1
Variability in human cytochrome P450 isozymes^a

Isozyme	Assay substrate	Variability (-fold)	Inducible	Evidence for polymorphism	Cancer impact
1A1	—	—	Yes	No	Smoking
1A2	Caffeine	50	Yes	No	PAH
2C	Mephenytoin	—	—	Yes (3% poor)	?
2D6	Debrisoquine	—	—	Yes	?
2E1	Chlorzoxazone	10	Yes	Yes	Halogenated hydrocarbons
3A4	Nifedipine	20	Yes	No	Aflatoxin-B1

^aGuengerich, 1989; Guengerich and Shimada, 1991.

carcinogens, and in the enzymes responsible for DNA repair, can also cause some individuals to be much more susceptible to carcinogenic chemicals than others (Harris, 1989).

The mixed function oxidase enzyme system, now referred to as cytochrome P450 (CYP), has frequently been associated with the toxicity and carcinogenicity of chemicals (Guengerich, 1989; Guengerich and Shimada, 1991; Guengerich et al., 1991). Studies have shown that the activity of the CYP enzymes can vary by more than a factor

of ten between individuals (Sabadie et al., 1980; Guengerich, 1989; Reitz et al., 1989), and that there is a genetic difference (polymorphism) between individuals with high activity and low activity that is associated with a different susceptibility to cancer (Uematsu et al., 1991). Genetic polymorphisms of the CYP enzymes across racial and ethnic groups have been observed (Stephens et al., 1994), as have quantitative differences in metabolic capacity (Shimada et al., 1994). Non-invasive methods have been developed to deter-

Table 2
Variability in human glutathione transferase isozymes

Isozyme	Assay substrate	Variability	Inducible	Evidence for polymorphism	Cancer impact
Alpha (GST2)	Cumene hydroperoxide ^a	—	—	No ^b	Aflatoxin-B1 ^c (detoxification)
Mu (GST1)	<i>trans</i> -Stilbene oxide ^a	—	—	Yes ^d (40% deficient)	Aromatic epoxides ^a (detoxification) deficiency in lung ^d , stomach ^e , and colon ^e
Pi (GST3)	Ethacrynic acid ^a	"Wide" ^b	Yes ^a	No ^a	Benzo[<i>a</i>]pyrene ^a (detoxification) expressed in tumors ^f
Theta	1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)-propane ^a	—	—	Yes ^h (30% deficient)	Halomethanes ^g (deactivation)

^aMannervik and Danielson, 1988; ^bBoard et al., 1990; ^cSimula et al., 1993; ^dZhong et al., 1991; ^eStrange et al., 1991; ^fHowie et al., 1990; ^gMeyer et al., 1991; ^hThier et al., 1993.

mine the activity of an individual's CYP enzymes by measuring the metabolites of common drugs in the urine (Berode et al., 1990; Peter et al., 1990; Bock et al., 1994). Table 1 summarizes information on several members of the CYP enzyme family.

Another enzyme system of importance for the toxicity and carcinogenicity of chemicals is the glutathione transferase (GST) system (Coles and Ketterer, 1990). However, as opposed to CYP enzymes, which are often responsible for generating the toxic or carcinogenic metabolite, GST enzymes typically serve as an important route of detoxification (Mannervik and Danielson, 1988; Simula et al., 1993). In particular, deficiency of GST- μ isozymes has been associated with increased risk of cancer (Seidegard et al., 1986; Strange et al., 1991; Zhong et al., 1991). In contrast, for some chemicals such as methylene chloride and ethylene dibromide the induction of carcinogenicity actually appears to be associated with DNA adducts from reactive intermediates produced during metabolism by a different GST isozyme, referred to as GST- θ (Guengerich et al., 1987; Meyer et al., 1991). For the particular GST isozyme implicated in this case, there is evidence that a significant portion of the human population possesses no detectable activity at all (Hallier et al., 1990, 1993; Thier et al., 1993; Pemble et al., 1994) and are therefore at much lower, if any, risk for the effects of chemicals which result from metabolism by this isozyme. Table 2 summarizes information on several members of the GST enzyme family.

2.2.2. Environmental factors

The personal environment of an individual can also greatly modify their risk from a chemical. For example, drinking alcohol induces the activity of the same CYP 2E1 isozyme which plays the major role in the metabolism, and therefore the carcinogenicity, of chemicals such as vinyl chloride (Nakajima et al., 1990). Increased metabolism can also result from exposure to other environmental chemicals, such as the inducibility of CYP 1A1/2 by aromatics, or prescription drugs, such as the induction of CYP 2B1 by phenobarbital

(Nakajima et al., 1990). Even dieting can enhance the metabolism, and therefore the carcinogenicity, of environmental contaminants (Sato and Namajima, 1985). Sex- and pregnancy-related variations in P450 content can also lead to differences in the metabolic capacity for chemicals (Nakajima et al., 1992; Fletcher et al., 1994). For example, sex differences have been noted in the metabolism of trichloroethylene in the human (Nomiyama and Nomiyama, 1971), with females excreting a larger proportion of the acid metabolite and a smaller proportion of the alcohol metabolite than males.

The activity of some enzymes, such as GST- α and GST- π , can be detected in the fetus (Guengerich, 1989), while others, such as GST- μ and CYP 2E1, cannot (Bolt et al., 1980; Guengerich, 1989). These differences have important implications for the likelihood of fetotoxicity and developmental effects from chemicals. For example, it has been suggested that the fact that vinyl chloride was not a transplacental carcinogen in animal studies may be due to the lack of CYP 2E1 activity in the fetus (Bolt et al., 1980). Moreover, as shown in Table 3, it is approximately a week before rat pups achieve adult levels of this isozyme. Thus metabolic status may also have an impact on the probability of neonatal toxicity for some chemicals. There can also be significant changes in pharmacokinetics and metabolism in the elderly (Birnbbaum, 1987; Ritschel, 1988); as shown in Table 4, some chemicals, such as the chemotherapeutic methotrexate, are less effective-

Table 3
Metabolism of vinyl chloride vs. age in rats^a

Age (days)	Maximum metabolism (nmol/h/g)
0	8
1	19
2	51
3	65
4	62
8	92
Adult	111

^aBolt et al., 1980.

Table 4
Total clearance of selected drugs in elderly vs. young adults^a

Drug	Clearance ratio (elderly/young adult)
Acetaminophen	0.75
Aspirin	0.7
Ibuprofen	1.1
Methotrexate	0.6
Theophylline	0.8-1.3
Valproic acid	0.95
Warfarin	0.85

^aRitschel, 1988.

ly cleared by the elderly, while others, such as the analgesic ibuprofen, are not.

Other personal factors affecting the response of an individual to a chemical include size, weight, condition, fat content, and level of physical activity. These factors effect the individual pharmacokinetics (uptake, distribution, and elimination) of the chemical exposure (Wallace et al., 1989). For example, an individual with a large proportion of fat will absorb more of a chemical such as trichloroethylene, and retain it longer, than a lean individual. This longer storage increases the opportunity for metabolism to the carcinogenic species. Studies on normal human volunteers have shown significant variation in individual pharmacokinetic behavior, and it is clear that this variability in pharmacokinetic factors is an important component of the overall interindividual variability of susceptibility to the toxic effects of chemicals (Hattis et al., 1987).

There are still other factors such as disease and hormonal status which could also affect the individual risk from exposure to a chemical, either because of an impact on pharmacokinetics or metabolism, or due to other interactions. Estrogens, for example, have been associated with both increased risk (for breast cancer) and decreased risk (for colon cancer), and are also metabolized by the CYP system (Guengerich, 1989). Therefore, the possibility of interaction with environmental chemicals includes metabolic inhibition or induction, as well as tumor promotion or repression. Pharmacokinetic and metabolic differences alone cannot explain the overall inter-

individual variation in susceptibility observed in exposed populations (Hattis et al., 1987; Hattis and Silver, 1994). Clearly there are other, less well understood interindividual differences, both acquired (due to environmental exposures or disease states) and inherited (due to genetic differences) that are also important determinants of the individual risk for development of toxicity from exposure to a chemical. However, to the extent that we can quantitatively describe and evaluate pharmacokinetic and metabolic variation, it will become increasingly possible to estimate the range of risks in an exposed population and to identify the factors which put individuals at the greatest risk.

3. PBPK modeling of human variability in risk assessment

Non-cancer risk assessment traditionally relies on applied dose measures, such as concentration of a chemical in inhaled air or in drinking water associated with the threshold for a toxic effect. Safety factors are then incorporated in an attempt to address the uncertainties associated with extrapolating across species, dose, duration, and routes of exposure, as well as to account for the potential impact of variability of human response (Dourson and Stara, 1983). A major disadvantage of this approach is that applied dose measures ignore fundamental pharmacokinetic processes which cause the relationship between applied dose and effective tissue dose to be complex and non-uniform across dose levels, dose routes, and species. A more biologically defensible risk assessment approach would be one in which an internal measure of effective tissue dose appropriate to each toxic endpoint was used. The internal dose associated with no-effect and low-effect levels in animals could be compared with those for potential human exposure scenarios.

While noncancer risk assessment makes use of an uncertainty factor (typically 10-fold) to attempt to account for the variability of human response, cancer risk assessment as currently practiced does not explicitly consider interin-

dividual variability in setting an acceptable human exposure level. Cancer potency estimates and risk specific doses reflect an estimate of the average risk to a population, and do not provide any information on the extent to which the risk to an individual could differ from the population average.

Physiologically based pharmacokinetic (PBPK) modeling provides a methodology both for considering pharmacokinetic differences across species when estimating human risk from animal data, and for evaluating the impact of pharmacokinetic variability on the dispersion of individual risks. Advantages of applying PBPK modeling in risk assessment have been discussed both for cancer (Clewell and Andersen, 1985, 1989; NRC, 1987; EPA, 1989; Frederick, 1993) and noncancer endpoints (Reitz et al., 1988a; Beck et al., 1993; Clewell and Jarnot, 1994). In addition, the use of PBPK modeling has been recommended to improve route-to-route extrapolation (Gerrity and Henry, 1990) and the estimation of risk for chemical mixtures (Mumtaz et al., 1993). A number of excellent reviews have been written on the subject of PBPK modeling (Himmelstein and Lutz, 1979; Gerlowski and Jain, 1983; D'Souza and Boxenbaum, 1988; Leung, 1991). Briefly, PBPK modeling attempts to describe the relationship between external measures of applied dose (e.g. amount administered or concentration in food, water, or air) and internal measures of delivered dose (e.g. amount metabolized or concentration in the tissue displaying the toxic response), using as realistic a description of mammalian physiology and biochemistry as is necessary and feasible.

Simple pharmacokinetic approaches have occasionally been used by regulatory agencies in cancer risk assessment; for example, the use of metabolized dose for trichloroethylene (EPA, 1985, 1987a). However, the only case to date where an agency has used a full PBPK approach was the EPA's use of the model of Andersen et al. (1987) in its latest revision of its inhalation risk for methylene chloride (EPA, 1987b). Application of the PBPK model for methylene chloride in a cancer risk assessment for occupational exposure has also been described (Clewell, 1993,

1995; Dankovic and Bailor, 1993), and is currently under consideration by OSHA (OSHA, 1991). Cancer risk assessments using PBPK models have also been described for perchloroethylene (Hattis et al., 1986; Chen and Blancato, 1987; Koizumi, 1989; Gearhart et al., 1993), ethylene oxide (Hattis, 1987), butadiene (Hattis and Wasson, 1987), ethylene dichloride (D'Souza et al., 1987), trichloroethylene (Koizumi, 1989; Fisher and Allen, 1993; Clewell et al., 1994a, 1995a,b), vinyl chloride (Chen and Blancato, 1989; Clewell et al., 1995b,c), dioxane (Reitz et al., 1990a; Leung and Paustenbach, 1990), chloroform (Reitz et al., 1990b), and ethyl acrylate (Frederick et al., 1992). PBPK models have also been applied in noncancer risk assessment (Clewell and Jarnot, 1994; Clewell et al., 1995d), as well as to derive accurate Biological Exposure Indices for workplace exposure monitoring (Leung and Paustenbach, 1988; Perbellini et al., 1990; Leung, 1992).

3.1. Uncertainty

One of the little recognized benefits of PBPK modeling in risk assessment is its ability to quantify the impact of pharmacokinetic uncertainty and variability. As it relates to the issue of using PBPK modeling in risk assessment, uncertainty can be defined as the possible error in estimating the "true" value of a parameter for a representative ("average") animal or human. Variability, on the other hand, should only be considered to represent true interindividual differences. Understood in these terms, uncertainty is a defect in knowledge which can typically be reduced by experimentation, and variability is a fact of life which must be considered regardless of the risk assessment methodology used. One of the attractive features of PBPK modeling is that it identifies those areas of uncertainty which are of the greatest importance for a specific risk assessment and which deserve further investigation. For example, PBPK modeling identified the need to measure human GST activity in order to refine the risk estimate for methylene chloride (Andersen et al., 1987; Reitz et al., 1988b, 1989). Several investigators (Farrar et al., 1989; Portier and Kaplan, 1989; Bois et al., 1990; Clewell, 1993, 1995) have attempted to estimate the overall

impact of parameter uncertainty in PBPK models on risk assessment predictions using the Monte Carlo method.

3.2. Variability

In the same way that it helps to illuminate uncertainty in the risk assessment process, PBPK modeling can also help to elucidate the potential impact of human variability on the internal exposure of an individual, as opposed to that of the average population. In a series of publications, Sato and coworkers have described the use of a simple PBPK model to study the kinetics of TCE and its metabolites in humans (Sato et al., 1977), to evaluate the impact of changes in physiological factors (Sato et al., 1991a) and environmental factors (Sato, 1991) on the kinetics of TCE in the human, and to predict the effects of interactions with ethanol consumption on TCE kinetics (Sato et al., 1991b). Other studies have also used PBPK models for investigating the impact of variations in the physiological or biochemical parameters on the internal exposure to a volatile chemical (Fiserova-Bergerova et al., 1980, 1984; Sato and Nakajima, 1987; Opdam, 1989a; Droz et al., 1989a,b), and for calculating the respiratory input of a volatile chemical from inhalation exposures (Opdam, 1989b; Opdam and Smolders, 1989).

3.3. Linking pharmacokinetic and metabolic variability to risk with PBPK models

The quantitative framework provided by a PBPK model makes it a simple matter to investigate the impact of differences in pharmacokinetic factors on personal risk. For example, a higher breathing rate and cardiac output should be used for occupational exposures involving exertion than for more sedentary occupations (Astrand and Rodahl, 1970). Using a PBPK model for methylene chloride developed by Andersen et al. (1987), Dankovic and Bailor (1993) were able to demonstrate that workers exposed to methylene chloride under light working conditions were at more than twice the risk estimated for resting individuals (Table 5). They also exercised the model to evaluate the risk implications of the heterogeneity of human metabolic activity.

Table 5
Effect of activity on pharmacokinetic parameters and relative cancer risk^a

Parameter	Flow rate (l/min)	
	Rest	Light work
Alveolar ventilation	7.0	17.4
Cardiac output	5.2	8.4
Liver blood flow	1.6	1.7
Relative lung cancer risk ^b	1	1.8

^aDankovic and Bailor, 1994.

^bBased on the PBPK model of Andersen et al., 1987.

Table 6 shows the *in vitro* cytochrome P450 and GST metabolic activities obtained for human liver samples from four individuals. Again using the PBPK model of Andersen et al. (1987), Dankovic and Bailor (1994) calculated that if those four individuals were exposed to methylene chloride, their relative risk of lung cancer would range from zero (for the individual lacking any GST activity for methylene chloride) to 50% greater than the average population risk estimate (Table 6, last column).

3.3.1. Sensitivity analysis

To the extent that a particular PBPK model correctly reflects the pharmacokinetic and metabolic processes underlying the delivery of the active form of a chemical to the target tissue, PBPK modeling also provides a means for ident-

Table 6
Variation in relative cancer risk resulting from variation in human metabolic activity for methylene chloride

Individual	P450 activity ^a (nmol/min/mg)	GST Activity ^a (nmol/min/mg)	Relative risk ^b (GST, lung)
99	5.27	2.62	1.35
103	1.53	0.0	0.0
105	13.0	2.71	1.10
109	6.24	3.03	1.52

^aReitz et al., 1989; ^bDankovic and Bailor, 1994.

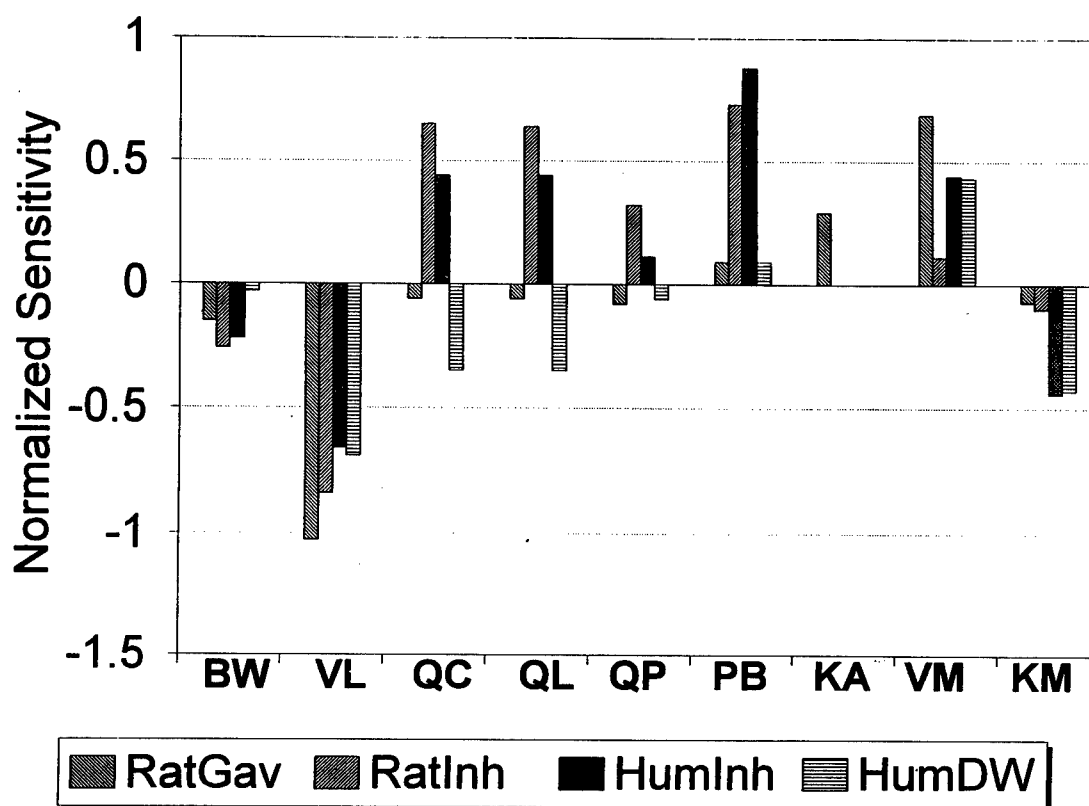


Fig. 1. Normalized analytical sensitivity coefficients for the parameters in the PBPK model for vinyl chloride, based on model predictions of the amount of vinyl chloride metabolized per liver volume per day. A sensitivity coefficient of 1 indicates that a 0.1% increase in the parameter produces a 0.1% increase in the prediction. Abbreviations: BW, body weight; VL, volume of liver; QC, cardiac output; QL, relative liver blood flow; QP, alveolar ventilation; PB, blood/air partition coefficient; KA, oral uptake rate; VM, capacity of metabolism; KM, affinity of metabolism; RatGav, rat oral gavage (12 mg/kg/day); RatInh, rat inhalation (50 ppm, 4 h/day); HumInh, human inhalation (1 ppm, continuous); and HumDW, human drinking water exposure (1 ppm, 2 l/day).

ifying the important physiological and biochemical parameters affecting individual risk. Fig. 1 shows the results of a sensitivity analysis performed on a PBPK model for vinyl chloride (Clewell et al., 1995c). The analytical sensitivity coefficients shown in the figure have been normalized, that is they represent the fractional change in output associated with a fractional change in the input parameter. For example, if a 0.1% change in the input parameter resulted in a 0.2% change in the output, the sensitivity coefficient would be 2.0. The model output in this analysis was the total amount metabolized, divided by the volume of the liver; this is the

target tissue dose metric used to perform a risk assessment for the liver effects (cancer or toxicity) of vinyl chloride. Parameter sensitivities are complex functions of the exposure scenario and animal being modeled, and can also vary over time (Clewell et al., 1994b). In this case the normalized sensitivities for four different exposure scenarios are shown: oral gavage of rats (RatGav), inhalation exposure of rats (RatInh), human inhalation exposure (HumInh), and human drinking water exposure (HumDW). Parameters with normalized sensitivity coefficients of less than 0.1 in absolute value were omitted from the figure for clarity.

Note that none of the parameters display sensitivities significantly greater than 1.0, indicating that there is no amplification of variability from the inputs (pharmacokinetic parameters) to the output (risk). This is, of course, a desirable result, since it indicates that small variations in pharmacokinetics do not lead to wide differences in individual risk. It can also be seen that of the 24 parameters in the vinyl chloride model, only eight have a significant impact on risk predictions based on any of the dose metrics: the body weight (BW), relative liver volume (VL), cardiac output (QC), relative liver blood flow (QL), alveolar ventilation (QP), blood/air partition coefficient (PB), oral absorption rate for oral gavage (KA), and the capacity (VM) and affinity (KM) for metabolism by CYP 2E1.

Thus, the results of this sensitivity analysis indicate that the most important pharmacokinetic parameters affecting the human risk from vinyl chloride exposure are:

- the blood/air partition coefficient (PB) and pulmonary ventilation (QP), which determine the uptake and elimination of vinyl chloride in the lungs,
- the total blood flow (QC), the relative blood flow to the liver (QL), and the capacity and affinity of metabolism by CYP 2E1 (VM and KM), which determine the rate of metabolism, and
- the body weight and relative volume of the liver (BW and VL), which determine the volume of distribution for the reactive metabolites produced by CYP 2E1.

All of these parameters can be reasonably well characterized from experimental data in both the animal and the human, so the impact of pharmacokinetic model uncertainty on the population average risk estimates should be small compared to other sources of uncertainty in the risk assessment process. However, the sensitivity of the risk predictions to the human values of some of these parameters also implies that the risk from exposure to vinyl chloride could vary considerably from individual to individual, depending on their specific physiology, level of

activity, and metabolic capability, as demonstrated above for methylene chloride.

3.3.2. Monte Carlo analysis

An estimate of the contribution of variability in pharmacokinetics and metabolism to human interindividual variability in susceptibility can be obtained from Monte Carlo analysis, in roughly the same way that variability in exposure assessment is evaluated by this technique. In this case, the PBPK model is run with parameter values sampled from distributions chosen to reflect the observed variation in each pharmacokinetic parameter in the human population. For example the distribution for VM, the capacity of CYP metabolism, is based on the reported variation in CYP 2E1 (Sabadie et al., 1980; Reitz et al., 1989; Guengerich, 1989), while the distribution of PB, the blood/air partition coefficient, is based on repeated determinations for two chemicals, PERC (Gearhart et al., 1993) and chloropentafluorobenzene (Clewell and Jarnot, 1994). The distributions of the physiological parameters were obtained from the physiological literature (ILSI, 1994). In the analyses described here, the parameters were assumed to be independent; a more accurate estimate of variability could be obtained if the correlations among important parameters were treated explicitly (Allen et al., 1996). Each time the model is run with a sampled set of parameter values, the appropriate dose metric for the toxicity of interest is output and the resulting risk estimate (for a cancer endpoint) or acceptable exposure (for a noncancer endpoint) is calculated. This process is repeated a large number of times (typically 200 to 500), generating the distribution of risks or acceptable exposures for the simulated population.

The results of this analysis for four chemicals are shown in Table 7. The variability of cancer risk estimates was estimated for three chemicals and five human exposure scenarios: lung tumors from occupational exposure to methylene chloride at the proposed OSHA Permissible Exposure Level (25 ppm), and liver tumors from lifetime continuous exposure to trichloroethylene or vinyl chloride in air or drinking water. Cancer risks varied by roughly 3- to 12-fold from the 5th to

Table 7
 Predicted distribution of risks and effect levels in a population due to human variability in pharmacokinetics and metabolism

Chemical/effect	Dose metric	Risk metric	Percentile in population		
			5%	50%	95%
<i>Cancer effects</i>					
Methylene chloride ^a / lung cancer	Amount metabolized by the glutathione transferase pathway/volume of liver	Risk (per thousand) for an occupational exposure to 25 ppm:	0.15 (0.0)	0.5	1.9
Trichloroethylene ^b / liver cancer	Trichloroacetic acid area under the curve	Risk (per million) for 1 ppb inhalation:	11	33	82
		Risk (per million) for 1 µg/l in drinking water:	0.4	1.7	4.0
Vinyl chloride ^c / liver angiosarcoma	Amount metabolized/ volume of liver	Risk (per million) for 1 ppb inhalation:	3.5	6.8	10.8
		Risk (per million) for 1 µg/l in drinking water:	0.64	1.14	1.63
<i>Noncancer effects</i>					
CPF ^d /liver toxicity	CPF area under the curve in the liver	No-observed-adverse-effect level human equivalent conc. (ppm):	2.6	1.8	1.1
Vinyl chloride ^e / liver toxicity	Amount metabolized/ volume of liver	Lowest-observed-adverse-effect level human equivalent conc. (ppm):	27	19	10

^aClewell, 1995; ^bClewell et al., 1994a; ^cClewell et al., 1995c; ^dClewell and Jarnot, 1994; ^eClewell et al., 1995d.

the 95th percentile. In the case of methylene chloride, the range of risks is actually for the 70% of the population possessing GST- θ activity, since the risk for the 30% of the population lacking this enzyme appear not to be at risk from this effect. The variability in the acceptable human exposure for a noncancer effect is shown for two cases: the human equivalent to the animal no-observed-adverse-effect level for liver toxicity from chloropentafluorobenzene and the human equivalent to the animal lowest-observed-adverse-effect level for liver toxicity from vinyl chloride. In both cases, the 5th and 95th percentiles of the simulated population differ by slightly under 3-fold. These results are consistent with the results of previous studies of human pharmacokinetic variability (Hattis et al., 1987; Hattis and Silver, 1994), which concluded that while pharmacokinetic variation was an important component of the observed interindividual variability of susceptibility, other factors must also play a significant role.

4. Conclusion

Standard carcinogenic risk assessment calculations estimate chemical potency for an "average" person, while noncancer risk assessments typically apply an uncertainty factor of 10 to account for interindividual variability. In applying the results of a risk assessment to an entire population, no quantitative estimate of the impact of intrapopulation variability on the potency of a specific chemical effect can be made unless a methodology is used which incorporates the factors which modulate individual response. For example, the personal risk for many chemicals is critically dependent on the individual's metabolic capability, but the standard risk assessment process is poorly designed to incorporate this information. One of the important contributions of PBPK models to chemical risk assessment is that they help to make key pharmacokinetic factors in the process more explicit, and provide a means for estimating the significance of these factors in

the final risk estimate. The impact of human pharmacokinetic heterogeneity on the range of individual risks, as compared to the average population risk, can be estimated by performing a Monte Carlo analysis of the effect of the variation of human metabolic and physiological parameters on the risks estimated with a PBPK model. The results of this analysis indicate that pharmacokinetic variability is an important, but not the only, component of the observed interindividual variability of human susceptibility to chemical toxicity.

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Interindividual variations in susceptibility and sensitivity: linking risk assessment and risk management

R. Julian Preston

Chemical Industry Institute of Toxicology, 6 Davis Drive, Research Triangle Park, NC 27709, USA

Abstract

In the past few years, our knowledge of mammalian genomes has increased enormously. Our understanding of the molecular basis of the normal cellular processes of DNA replication and repair and cell cycle control, together with how their fidelity malfunctions as part of tumor development, has increased in parallel. This has led to a clearer appreciation that there are subpopulations that have been generically described as being genetically or otherwise susceptible to the induction of cancer or birth defects. The term *susceptibility* is a *default option*, since there clearly will be a very broad range of sensitivities among the so-called susceptible populations, dependent upon the specific underlying mechanism. This could lead to the conduct of risk assessments for each specific situation, involving both genotypes of individuals and agents of concern. This would ideally take into account the effects on response of various modifying factors, genetic and other. One advantage to be gained from this approach is the ability to determine if a particular susceptibility places subpopulations at extreme risk as compared to the overall normal distribution of risk in the population, or whether such a susceptible population presents a slight extension of the upper bound of the risk distribution or lies within the normal distribution. In addition, the specific mechanism of the susceptibility as related to exposure scenarios and the magnitude and demographics of the susceptible populations need to be taken into account. Thus, the management of risk has to be linked to the specific risk assessment. For many of the so-called susceptible populations an uncertainty factor of less than 10, even including 1, would be predicted to bring the risk within the normal distribution. It is hoped that as more mechanistic information on susceptibility becomes available and a specific risk can be defined, the practice of risk management will be considerably improved.

Keywords: Genetic susceptibility; X-rays; Ultraviolet light; Mutagenic chemicals; Safety factors

1. Introduction

The issue of how to account for interindividual variations in susceptibility and sensitivity to cancer induction, especially where this has

a genetic basis, has generally been treated in a generic way for risk management purposes. There is a tendency to combine all potential or known sensitivity traits into a single category, and suggest that a single safety factor (default option) be applied to *protect* such individuals from the adverse health effects of environmental or occupational exposures to chemicals and/or radiations.

* Corresponding author, Tel.: +919 558 1367; Fax: +919 558 1300; E-Mail: Preston@CIIT.org.

Such an approach will more than likely provide an overestimate of allowable exposure in some cases and an underestimate in others. The information presented in this chapter is an attempt to show how a unique risk assessment process and risk management decision will need to be developed for each particular combination of susceptibility gene and agent of exposure. The discussion will, by necessity, be only scratching the surface of a very complex problem, but it is hoped that it will provide an impetus toward the development of a rational approach to risk assessment for genetically susceptible/sensitive populations. Since it is a concept paper it contains relatively few references, but it is hoped that this will not detract from its utility.

2. Susceptibility and sensitivity: what to do with scientific knowledge

For the purposes of the present discussion the emphasis will be on tumors as an endpoint and cancer risk assessment and management as the process to be developed. It will probably be apparent that other noncancer endpoints such as birth defects, and adverse neurological, developmental and reproductive outcomes will be the subject of similar deliberations. In this context it is important to note that there is a distinction between interindividual susceptibility and sensitivity, one that should become apparent in the subsequent section. For cancer, a genetic *susceptibility* is indicated by an increase in the *background* frequency of a specific tumor type or tumors overall. A susceptible sub-population will carry a specific genetic alteration (or genotype) compared to the mean of the general population. In contrast, a genetic *sensitivity* is indicated by an increase in a specific tumor type or tumors overall *in response to an environmental stress*. Such stress might typically be exposure to certain chemicals or exposure to ionizing radiation or ultraviolet light. This distinction becomes of particular importance when expressing any added risk from exposure in terms of background frequency of tumors.

Having developed this working definition of

susceptibility and sensitivity, it is necessary to determine what one would do with the knowledge that a particular population subgroup has an increased susceptibility and/or sensitivity to carcinogenesis. An increased susceptibility would indicate the potential for increased sensitivity, but any risk assessment or risk management decision would be made in the absence of knowledge of response to a particular exposure and would probably utilize only a default approach. This would incorporate the use of somewhat arbitrary, albeit conservative safety factors. An observed or predicted increased sensitivity, on the other hand, could be incorporated directly into a risk assessment for the particular sensitive population for comparison with the general population. Attention should be given to information on the possible role of a specific genotype in the multistage cancer process. A risk management decision would need to be attentive to the relative magnitude of the sensitivity in relation to the distribution of sensitivities in the general population, the estimated or known size of the sensitive population, and the likelihood of the sensitive group receiving a specific exposure.

The aim of the risk management process with the inclusion of sensitive populations is clearly expressed by the Committee on Risk Assessment of Hazardous Air Pollutants in *Science and Judgment in Risk Assessment* (NRC, 1994, page 208):

It can be argued (*in addition*) that EPA has a responsibility, in so far as it is practicable, to protect persons regardless of their individual susceptibility (sensitivity) to carcinogenesis (we use *protect* here not in the absolute, zero-risk sense, but in the sense of ensuring that excess individual risk is within acceptable levels or below a de minimus level).

Within this framework of protection, it is important to note that the descriptions of susceptibility and sensitivity are not based on unique values of an enhanced response, but rather on alterations in the distribution of response seen in the normal population, as described below. Thus, an approach for risk assessment, based upon this need for considering distributions of risk, was proposed by the Committee on Risk Assessment of Hazardous Air Pollutants (NRC, 1994, page 208):

EPA could develop a "default distribution" of susceptibility, and then generate the joint distribution of exposure and cancer potency [in light of susceptibility (*sensitivity*)] to find the upper 95th or 99th percentile of risk for use in a risk assessment.

This concept of distributions of risk is a recurring one throughout the present chapter, since it provides a most important foundation to the development of rational risk management approaches for susceptible/sensitive populations, including the means for incorporating a wide range of different types of susceptibility and sensitivity.

The previous discussions are obviously predicated on the conclusion that it is essential that a much clearer understanding be obtained of the basis for interindividual variations in susceptibility and sensitivity at the cellular and molecular level in order to provide more reliable default factors or to develop distributions of risk. In some cases, there is an initial understanding of underlying mechanisms, and progress clearly is rapid (Cold Spring Harbor Symposia on Quantitative Biology, 1994).

Despite the need for mechanistic data on the underlying basis for genetic susceptibilities and sensitivities, there will remain a necessity to be cautious about the interpretation of the data and the extension into general rules. Examples of pitfalls to the use of mechanistic data are presented in Genetic susceptibility and sensitivity: what are we looking for? (Section 5).

3. The genetics of susceptibility and sensitivity

In order to incorporate the effects of genetic susceptibility and sensitivity into risk assessment models and risk management practices, it is desirable to determine the extent of the concern. As a general rule, most of the susceptibility or sensitivity factors (e.g. genotypes) that are common in the population tend to increase relative risk by small amounts. Those conferring high relative risk are present at a low frequency; this is particularly true for susceptibility for which background frequencies of cancer, for example, are high. The question of paramount importance for risk management would, therefore, seem to be, "What do we know about relatively *common* factors that might increase sensitivity by a rela-

tively large amount?" In other words, would exposure to a particular chemical produce a significant increase in cancer risk to the sensitive group?

Largely as a consequence of the fact that a cell has an intricate set of controls for maintaining genomic and cellular integrity, there will be the opportunity for mutations at any one of these controls to jeopardize this integrity in one or more ways. The fact that this happens relatively rarely (or at least as evidenced by cancer frequency, for example) is probably a consequence both of genetic redundancy, whereby more than one gene can control a single function, and the ability of cells to undergo various types of repair, particularly at the DNA level. However, the probability of producing a mutation in genes controlling genomic and cellular integrity can be enhanced in a number of ways, any of which can give rise to an increase in individual sensitivity or susceptibility to cancer (or other adverse health outcome). While the following list is probably not all-inclusive, it covers the majority of factors influencing individual sensitivity.

The dose of an exogenous agent will clearly influence sensitivity to mutation induction. For the present discussion the role of genetic controls of target cell dose will be the area of focus. It is appreciated however that, in terms of target tissue dose a sensitive group can also be considered to be one receiving a high exposure. Such situations have been covered in other chapters in this volume.

There is a broad range of genetic polymorphisms in humans for xenobiotic metabolizing and detoxifying enzymes, some of which have been correlated with the frequency of a specific tumor type. For example, the slow and rapid acetylation of aromatic amines have been implicated as cancer risk factors. The slow one has been associated with bladder cancer in individuals exposed to *N*-substituted aryl compounds in the dye industry (Cartwright et al., 1982) and the rapid one with colon cancer (Lang et al., 1986; Ilett et al., 1987; Ladero et al., 1991). In a somewhat different vein, the GSTT1 and GSTM1 polymorphisms (and the homozygous null phenotypes) appear to control a relative sensitiv-

ity to sister chromatid exchange induction in cultured lymphocytes exposed to diepoxybutane (DEB). In broad terms, DEB-sensitive individuals are of the GSTT1 null phenotype, with there being no influence from the GSTM1 genotype (Norppa et al., 1995). It remains exceedingly difficult to provide strong evidence for a causative relationship between a proximate event to exposure, namely metabolism, and a distal event, cancer. It should also be noted that assigning relative risk factors based on a single genetic polymorphism is perhaps begging the question in overall terms since there are multiple metabolic pathways and detoxification processes for any particular chemical, all of which are under genetic control.

It is difficult to determine if interindividual differences in levels of DNA damage are due to differences in dose, differences in DNA repair, or to real differences in production of DNA damage. It has been shown in molecular epidemiology studies that there can be as much as a 150-fold range in DNA adduct frequency among exposed individuals (Harris, 1989). However, in general the range of frequencies of adducts among individuals is unimodal, perhaps indicating the absence of genetically sensitive populations for DNA damage induction itself.

There are essentially two outcomes for a DNA adduct (or other forms of DNA damage); it can be repaired by one of the several DNA repair pathways, or it can be "replicated" by being present in the template at the time of DNA replication. The timing of the events of DNA repair and replication is important for maintaining genomic integrity. There is a requirement for repair to precede replication in order to reduce errors of replication from a damaged DNA template. Thus, the rate of DNA repair and its fidelity will help govern the potential for mutation induction via misrepair and/or misreplication, and these characteristics will be genetically controlled. Recent advances in understanding DNA repair and replication through molecular biology techniques have enabled us to begin to appreciate the range and types of genetic susceptibility and sensitivity through these processes and even to identify individuals at potentially

increased risk. The next section will expand upon this discussion.

DNA replication is a complex process that requires the coordinated action of many enzymes. In addition, the timing of the whole process of replication has to be exquisitely controlled because of the multiple replicons that are required to replicate the genome in a few hours instead of the one year that a single replication would require (Su et al., 1995). It is more than likely that mutations in the genes involved in conducting and controlling replication will have a broad range of susceptibilities for background mutation frequency and sensitivity to exposure to mutagenic and some nongenotoxic chemicals. However, at this time there is little information to demonstrate the consequence of such errors at the organism level. Several sophisticated studies by Kunkel and colleagues (Thomas et al., 1991; Kunkel, 1992; Boyer et al., 1993) have demonstrated that a number of factors can influence DNA replication fidelity in *in vitro* systems, and alter mutation frequency. However, such information currently remains beyond the bounds of risk assessment.

In addition, it has been demonstrated that a number of human disorders, particularly with an irregular inheritance pattern, can arise from rapid expansion of trinucleotide repeat sequences within or outside coding sequences for specific genes (Sutherland and Richards, 1995). This appears to be due to replication errors as a consequence of polymerase "stuttering". Alterations in the replicative polymerase could enhance the probability of this occurring.

The recent cloning of a number of genes associated with DNA repair has led to an enormous expansion of our understanding of the process. For example, several genes have been cloned that are mutated in xeroderma pigmentosum (XP) patients, and these patients show increased sensitivity to ultraviolet light (UV) and the subsequent development of skin cancer. These genes are, in fact, also associated with transcription (for short review see Sancar, 1994). This finding also provided an explanation of how pyrimidine dimers are more rapidly repaired in the transcribed strand of actively transcribed genes than

in other regions of the genome (for short review see Hanawalt, 1994; Chalut et al., 1994). At least 12 proteins are required for nucleotide excision repair (NER), as shown by the elegant *in vitro* reconstitution studies of Aboussekhra et al. (1995). Thus, one would predict that there would be a wide range of DNA repair capacities based upon the possibilities for mutation in any one of the genes involved in NER. Some will produce major effects like XP, but others could be expected to produce minor effects that might have gone undetected.

What would be the predicted outcome from a reduction in DNA repair rate? For ultraviolet light, this can be surmised from the experiments of Tornaletti and Pfeifer (1994). A summary of their finding is given in Fig. 1. They showed that the hot spots for mutation in the p53 gene following exposure to UV were at sites of slow repair of pyrimidine dimers. Only some of these sites were also hot spots for DNA damage. Thus, a slowing of repair for a variety of different types of DNA damage could increase the mutation frequency. It is these somewhat more subtle effects of mutations in required cellular processes that have to be considered of particular significance for genetic susceptibility, and the magnitude of the sensitivity from exposure to exogenous agents will determine the risk management approaches (see Section 4).

Similar arguments to those made for sensitivity as a result of alterations in NER hold for other repair processes. For example, it has recently been shown that mutations in one or more genes

involved in mismatch repair can lead to genetic instability and cancer, at least for hereditary nonpolyposis colorectal cancer (for short review see Modrich, 1994). This finding has led to the concept of a mutator phenotype (perhaps mutator genotype is more appropriate), whereby a cell that is mutant for a specific housekeeping function would be particularly sensitive to the induction of mutations in the cancer pathway. This type of genetic alteration is perhaps at the root of genetic susceptibility and sensitivity (Parsons et al., 1995).

For the purpose of the present discussion of what constitutes genetic susceptibility and sensitivity, the last of the cellular processes to be considered that will affect this is accurate control of the cell cycle. It has already been mentioned above that a critical component of sensitivity to mutation induction by DNA damaging agents is the relationship between DNA repair and replication. In order to insure that maximal repair of DNA damage takes place before replication is initiated and before the cell enters mitosis, the cell has developed an intricate system of checkpoints most clearly described at the G₁/S and G₂/M boundaries (Weinert and Lydall, 1993; Cox and Lane, 1995). The prototypical cell cycle checkpoint gene is p53. In simple terms, p53 serves to recognize DNA damage (assumed to be DNA strand breaks) and binds to this to initiate DNA repair (Bakalkin et al., 1994). At the same time, it serves as a transcription factor to increase expression of p21, a cell-cycle protein, that inhibits replication by binding to PCNA (Li et al., 1994). The DNA repair function of PCNA is not inhibited by p21. The DNA-damaged induced protein Gadd45 is also up-regulated by p53, and it may stimulate excision repair and inhibit cell replication (Smith et al., 1994). In this way the cell cycle is checked at the critical G₁/S boundary and DNA repair is stimulated. p53 itself may well be involved in DNA repair (Wang et al., 1995). p53 is frequently mutated in human and to a lesser extent rodent tumors, suggesting that loss of cell cycle checkpoint function can contribute to genetic instability (Livingstone et al., 1992). Also, individuals with a germ line mutation in p53 (Li-Fraumeni) are susceptible to the develop-

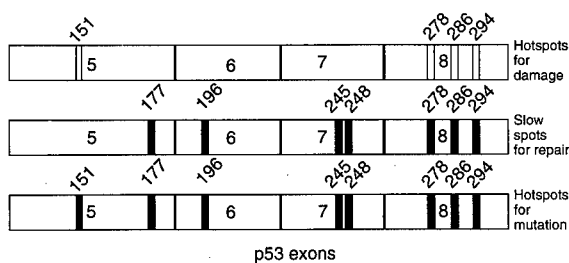


Fig. 1. A diagram of the p53 gene depicting the hotspots for ultraviolet light-induced DNA damage, the slow spots for DNA repair, and the hotspots for mutation induction (from Service, 1994).

ment of a range of tumors (Malkin et al., 1990).

It is, in some ways, interesting to contemplate why the p53 checkpoint function has been "developed" in response to DNA damage from ionizing radiation and similarly acting agents, when repair of DNA strand breaks is relatively rapid and the need for checking prior to replication less critical. In fact, chromosomal alterations and mutations induced by ionizing radiations in cells irradiated in the G₁ phase and in unreplicated DNA in the S phase are the product of misrepair of DNA damage (Preston, 1995). It remains of particular importance to understand any role of p53 in response to DNA adducts and further to determine if other genes are involved in checking (recognition) and repair for these DNA damages.

There appear to be other associations between cell cycle checkpoints or control and DNA repair. For example, in yeast there is a G₂/M checkpoint that involves the MEC3 and rad24 genes (Lydall and Weinert, 1995) and the recently cloned ataxia telangiectasia (AT) gene has a sequence similarity to phosphatidylinositol-3' kinases that are involved in mitogenic signal transduction and cell cycle control (Savitsky et al., 1995).

Again, it is apparent that there is a wide range of cell cycle control genes that can be mutated, resulting in the potential for increased sensitivity to mutation induction that might ultimately translate into increases in cancer risk. In order to combat this, cells have developed a degree of redundancy to protect against damaging effects from alterations in housekeeping gene function. It is difficult at this time to determine what the consequences are of only small changes in cell cycle duration or control. It is this aspect of genetic susceptibility and sensitivity that perhaps requires the greatest attention in the context of risk assessment and risk management. This will require a better understanding of distribution of risk and the potential for true outliers and the underlying mechanisms responsible for these outliers.

4. Genetic susceptibility/sensitivity and distribution of risk

When considering the impact of a genetically susceptible or sensitive subpopulation on the risk

assessment process, it is most important to describe this in the context of the normal distribution of risk in the population. For the purposes of the present discussion, it has been assumed that risk in the population is normally distributed. The location of the relative risk for different subpopulations in relation to the normal population will depend upon the particular genetic alteration conferring susceptibility and the agent of exposure resulting in increased sensitivity to tumor formation. The examples used stem from the information presented in the preceding section.

4.1. Genotypes that increase susceptibility and sensitivity

Individuals who are heterozygous for the p53 tumor suppressor gene develop a variety of tumors (Malkin et al., 1990). Similar data have been shown for mice that are heterozygous for a p53 "knockout" (Donehower et al., 1992), with p53 homozygous knockouts developing tumors (largely lymphomas) even earlier in life. Thus, p53 heterozygous individuals are cancer susceptible and their relative risk would be significantly increased over the normal distribution (Fig. 2). In addition, mice that are p53 +/– are much more sensitive than their wild-type counterparts to tumor induction by a variety of chemicals (Harvey et al., 1993). Thus, it is reasonable to predict that Li-Fraumeni individuals would be more

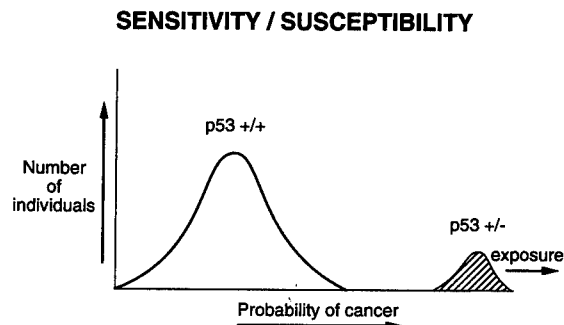


Fig. 2. Predicted distributions for cancer risk in the general population (p53 +/+) and in p53 +/- individuals for background (susceptibility) and induced (sensitivity) risk. Upper case for SUSCEPTIBILITY and SENSITIVITY indicates that both are predominant responses.

sensitive than the average population to the induction of cancer from carcinogen exposure. The p53 +/- genotype represents an example of genetic susceptibility and sensitivity. It is predicted that the frequency of Li-Fraumeni genotypes is rare in the population, and so the concern for overall risk management would be relatively minimal.

4.2. Genotypes that increase sensitivity

Individuals who are homozygous for ATM (mutated in AT) are at an increased risk of developing cancer and are very sensitive to ionizing radiation. Such individuals are rare in the population (1 in 40 000 to 1 in 100 000) and are diagnosed phenotypically at an early age. Thus, they would not be a significant component in risk management. However, the individuals who are heterozygous for ATM could represent about 1% of the population. Even with the recent cloning of the ATM gene (Savitsky et al., 1995), it is very difficult to identify heterozygous individuals, except for the obligate ones (parents of AT children). It has been proposed that AT heterozygotes are at increased risk of developing cancers of several types, particularly breast cancer (Swift et al., 1991). It cannot be determined if this is a susceptibility or a sensitivity (to ionizing radiation). However, the reported increase remains the subject of controversy. If AT hetero-

zygotes are at increased risk, would that place their cancer risk beyond the range of the normal distribution (Fig. 3)? It could be argued that AT heterozygotes are more sensitive to cancer induction by ionizing radiation than homozygous wildtype, but this remains to be demonstrated. Some recent studies by Scott et al. (1994) utilizing an assay for G₂ sensitivity to radiation-induced chromosome aberrations indicated that about 42% of women with breast cancer showed a sensitivity that was similar to obligate AT heterozygotes. This does not serve to show that AT heterozygotes are more susceptible to radiation-induced breast cancer but that predisposing genes involved in processing DNA damage are more likely to be present in breast cancer patients and possibly causative, in part. What will be necessary is to determine the magnitude of increased sensitivity, the genes involved, and the specific agents that might enhance this sensitivity. With AT, for example, ionizing radiation is the agent of concern. This highlights the fact that for any particular genotype a risk assessment risk management approach has to be cognizant of the specific sensitivity, namely the agent involved.

4.3. Genotypes producing small increases in susceptibility and sensitivity

Under Sections 4.1 and 4.2. above, the genetic susceptibilities and sensitivities would be predicted to produce large increases in cancer risk, placing the response well beyond the normal

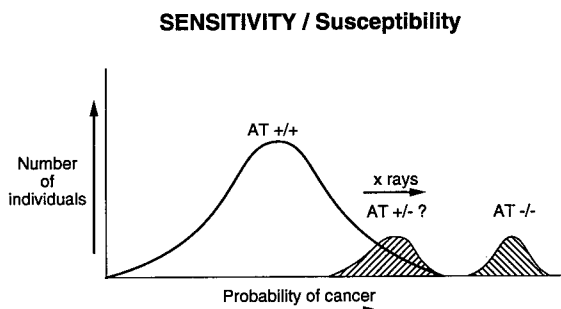


Fig. 3. Predicted distributions for cancer risk in the general population (AT +/+) and in AT -/- individuals for background and X-ray-induced risk. The background and X-ray-induced cancer risk for AT +/- is the subject of discussion (see text). Upper case for SENSITIVITY indicates that it is the predominant response.

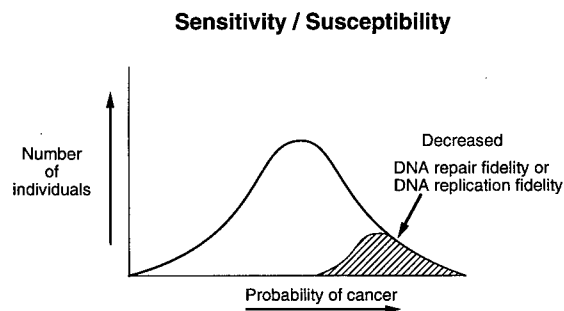


Fig. 4. A predicted distribution for cancer risk in the general population and for individuals with a decreased fidelity for DNA replication or repair. Lower case for susceptibility and sensitivity indicates that neither is the predominant response.

distribution of risk. In addition, such genotypes would be expected to be rare and the inducing agent specific so that risk assessments could be conducted for specific very minor subgroups on an as needed basis. Perhaps of greater concern are changes in necessary cellular processes such as DNA replication or repair that cause only minor increases in background mutagenicity but would, for example, result in cancer risk that is at the upper bound of the normal distribution (Fig. 4). The emphasis here is on genotypic alterations that would result in relatively small changes in DNA repair or replication fidelity, and does not include ones such as xeroderma pigmentosum or mismatch repair gene mutations (hMLH1 or hMSH2, for example). For these individuals the probability of sensitivity or susceptibility to cancer approaches 100%. In cases of more subtle changes in repair or replication kinetics or fidelity it is likely that there would be a small increase in sensitivity to exposure to mutagenic chemicals compared to wild-type genotypes, and a consistent increase in cancer. The magnitude of such a change is unknown, but the relative frequency of genes causing subtle changes might well be quite high. Thus, it is these genetic susceptibilities that need to be the subject of intensive study, and that should be of concern in the development of realistic risk assessment models. This will allow for "protective" risk management decisions to be made. It is the *increased sensitivity to risk* in the context of the *normal distribution of risk*, that is being "protected". This was considered to be the appropriate approach by the NRC (1994).

5. Genetic susceptibility and sensitivity: what are we looking for?

This final section will present three examples to highlight the fact that it is very difficult to predict genetic susceptibilities and sensitivities and in particular to devise general principles. Thus, it will be necessary to consider risk assessments for individual genotypes in their genomic context and in combination with exposure to a specific agent or defined class of agents that have a very similar mode of action.

5.1. Genes do not function in "splendid isolation"

In the previous section, it was noted that p53 serves as one of the cell cycle checkpoints and as a component of the accompanying DNA repair. In order to consider the potential impact of germ line p53 mutations on subsequent cancer risk, however, it is important to appreciate that p53 is a component of a complex signaling pathway or cascade. Several downstream elements such as p21 and Gadd45 have been identified, with upstream elements being less clearly defined (Cox and Lane, 1995). It has been proposed that the AT gene product acts upstream of p53, p21, and Gadd45 in response to gamma rays but not methyl methanesulfonate (J. Hall, referenced in Strike, 1995). This observation further emphasizes the fact that not only is there a number of genes acting in concert along the p53 cascade, but that this pathway has been largely defined for ionizing radiation. The response of p53-deficient cells and organisms to other agents is far less well understood.

It should also be noted that the genetic status of other genes in the p53 pathway, for example, will influence responses in individuals who are p53 heterozygotes. This same argument will hold for any number of pathways leading to cell cycle control, DNA repair, and signal transduction, for example. Until the complete pathways and various interactions are understood, it is necessary to use caution in predicting susceptibility and sensitivity from the presence of a single genotype in an individual or population.

5.2. Predicting responses from alterations in housekeeping genes

It would be expected perhaps that mutations in a range of housekeeping genes, such as those involved in DNA repair or replication, would produce similar increases in susceptibility to cancer induction, and that risk assessments could incorporate a single predicted susceptibility factor. While the information available on the effects of alterations in such housekeeping genes is not readily available, one comparison can serve to show that caution should be exercised in predicting one from another. Transgenic mice that are homozygous for the knockout of the DNA repair

gene, ERCC-1, have nuclear abnormalities of liver cells and die before weaning (McWhir et al., 1993). In addition, they have elevated levels of p53 as the cells attempt to handle the unrepaired endogenously induced DNA damage. Thus, the consequences of an absence of ERCC-1 are drastic, even in the absence of exogenous exposure.

In rather sharp contrast, there is normal development of mice that are homozygous for the knockout of (ADP-ribosyl) transferase and thus lack poly (ADP-ribosyl) action, a component of DNA repair and maintenance of chromatin structure (Wang et al., 1995). The only apparent phenotypic response is a susceptibility to skin disease in older mice, indicating a role for ADPRT in response to environmental stress. The impact of ADPRT mutations on genetic susceptibility and sensitivity is difficult to assess, and certainly hard to have predicted from the observations made for ERCC-1, another housekeeping gene, and vice versa.

5.3. *The comparative effects of different alterations in a single cellular process*

The third area that highlights the concept of how to predict genetic susceptibility is that of dissimilarity of response from alterations in separate components of one cellular process, particularly an essential one.

Three diseases in humans are associated with defects in nucleotide excision repair: xeroderma pigmentosum (XP), Cockayne's syndrome (CS), and trichothiodystrophy (TTD). In the case of XP, there are eight known complementation groups, XPA through G and ERCC-1. It has recently been shown that each of these codes for a protein involved in nucleotide excision repair, and that the majority are either part of or associated with the transcription complex TFIIH that forms part of the repair complex (Sancar, 1994). Mutations leading to loss of function in any of these genes gives rise to the XP phenotype which is characterized by neurological abnormalities and sunlight-induced photodermatitis including skin cancer.

CS appears to be caused by mutations in the CSA, CSB/ERCC6, XPB and XPD genes. These mutations lead to an uncoupling of transcription

and repair, such that there is deficient repair of UV-induced DNA damage in the transcribed strand of active genes (Van Hoffen et al., 1993). Patients with CS show photosensitivity, and growth and mental retardation but no increase in skin cancer.

TTD patients have mutations in XPB, XPD, and XPG genes and quite possibly other subunits of TFIIH or associated components of the repair complex. They typically have brittle hair, mental retardation and neuroskeletal abnormalities but again no increase in skin cancer and photosensitivity only in some cases (Stefanini et al., 1993).

It is of particular interest to note that these three syndromes involve mutations in the same cellular process and in some of the same genes, and yet the clinical manifestations are quite different. In fact, humans completely defective in nucleotide excision repair can survive into early adulthood quite normally in the absence of exposure to sunlight and other mutagenic agents.

In this example all three syndromes exhibit rather extensive clinical manifestations. However, it is feasible to expect that there could be a range of additional mutations in the NER complex that would present much less severe phenotypes (susceptibility) but that would have increased sensitivity to certain exposures. However, predicting the particular outcome and/or severity is extremely difficult, as exemplified by the above example for XP, CS, and TTD. This is an area where more research can only enhance predictability.

6. Conclusions

Despite the extraordinary recent advances in cellular and molecular biology that have enhanced our understanding of the basis for human disease, the approaches for incorporating this information into risk assessment and risk management activities are still in their infancy. In particular, determining how to incorporate the influence of genetic susceptibility and sensitivity into the process is singularly difficult. To do so is both critical and intractable.

Some general principles have been presented in this paper. The cellular activities that would affect susceptibility and/or sensitivity have been described, with the addendum that each should be considered separately, as should any agent of exposure. Risk should be considered as a series of distributions, one for the normal population and others for susceptible or sensitive subgroups. Increases in risk will then be a statistical comparison of distributions.

It is further emphasised that caution needs to be exhibited when using information obtained for one phenotype or cellular process and pragmatically assuming that the same response would be produced by mutations in similar processes or by different mutations in the same function.

Clearly the understanding of what constitutes genetic susceptibility and sensitivity is an evolving process, as is linking the information obtained to the science of risk assessment and the practice of risk management.

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