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MUSC
MEDICAL UNIVERSITY OF SOUTH CAROLINA

Environmental Biosciences Program
Third Quarter Report, Year 2
December 2004 — February 2005

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Principal Investigator

For

Cooperative Agreement DE-FC09-02CH11109

Submitted to the
U. S. Department of Energy

By The

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Table of Contents

1.0 Introduction

In May 2002, the United States Department of Energy (DOE) signed Assistance Instrument Number DE-FC09-02CH11109 with the Medical University of South Carolina (MUSC) to support the Environmental Biosciences Program (EBP). This funding instrument replaces DOE Assistance Instrument Number DE-FC02-98CH10902.

EBP is an integrated, multidisciplinary scientific research program, employing a range of research initiatives to identify, study and resolve environmental health risks. These initiatives are consistent with the MUSC role as a comprehensive state-supported health sciences institution and with the nation's need for new and better approaches to the solution of a complex and expansive array of environment-related health problems.

The intrinsic capabilities of a comprehensive health sciences institution enable MUSC to be a national resource for the scientific investigation of environmental health issues. EBPs success as a nationally prominent research program is due, in part, to its ability to task-organize scientific expertise from multiple disciplines in addressing these complex problems

Current research projects have focused EBP talent and resources on providing the scientific basis for risk-based standards, risk-based decision making and the accelerated clean-up of widespread environmental hazards. These hazards include trichloroethylene (TCE), polychlorinated biphenyles (PCBs), and low-dose ionizing radiation. A project is also being conducted in the use of geographical information system technology to analyze population health risks related to environmental hazards as a tool for risk-based decision-making.

Questions, comments or requests for further information concerning the activities under this cooperative agreement can be forwarded to Dr. Lawrence C. Mohr in the EBP office of the Medical University of South Carolina at (843) 792-1532.

1.1 Summary and Significance of Year Two Projects

Toxicology

- Trichloroethylene (TCE) is the most prevalent and widespread chemical contaminant at DOE sites. TCE is regulated as a human carcinogen based upon its hepatocarcinogenicity in a crude mouse model. Very little is known about the molecular mechanisms of carcinogenesis and the human health effects of TCE. MUSC has developed a comprehensive research program on the molecular mechanisms of disease pathogenesis and the human health effects of TCE to better understand the risks to workers at DOE sites. Through this research program, MUSC helps to ensure that TCE risk assessment and remediation activities are based upon sound science.

- PCBs and complex PCB mixtures are major environmental contaminants at DOE sites. Previous MUSC research has shown that complex mixtures of PCBs have immunotoxic effects on human lymphocytes and lymphocytes in laboratory mice. Previous work has also produced a method for the aerobic and anaerobic biodegradation of PCB mixtures by bacteria. Current research is underway to determine whether or not the bacterial biodegradation of complex PCB mixtures lowers toxicity to the immune system. This research is extremely important in demonstrating the usefulness of PCB biodegradation as a remediation technology that lowers human health risks.

Risk Assessment

- The adverse health effects of both ionizing and non-ionizing radiation are of concern to DOE and the public. Many important questions about the adverse human health effects of low-dose and low-dose rate radiation exposures remain unanswered – especially with respect to cancer risks. MUSC has developed a comprehensive research program for the study of the effects of low-dose and low-dose rate radiation exposures on human health.

- Population risk studies in areas surrounding DOE sites are of utmost importance to the department and to the citizens who live in these areas. The Savannah River Region Health Information System is a very important national, regional, and DOE resource for the study of population health effects in the area surrounding the Savannah River Site. In conjunction with the Savannah River Region Health Information System, MUSC has developed an extremely powerful Geographical Information System in which databases containing health, environmental, demographic and socioeconomic data can be integrated and analyzed for specific population health risks.

1.2 Program Expenditures

EBP Expenditure Summary Third Quarter

The table below reflects **expenditures** by budgeted category recorded for the period December 2004, through February 2005, and year-to-date, for Cooperative Agreement CH11109.

Budget Category	3rd Qrt.	YTD
	(Dollars in thousands)	
Personnel	$ 323	$ 960
Supplies	60	134
Travel	02	10
Other	07	14
Subcontract	26	155
Equipment	06	06
F & A	180	514
Total	$ 605	$ 1,794

2.0 Program Management and Development Office

The mission of the Program Management Office is to ensure that all projects of the cooperative agreement achieve their stated goals and objectives and are carried out in an efficient and cost-effective manner. The executive leadership of the program has adopted a strategy-focused management approach that carefully aligns the resources and core competencies of the program with research priorities developed in coordination with DOE. Specific Program Management responsibilities include workplan development, budget formulation, task organization of multidisciplinary research teams, financial management, progress reporting and program review.

The Program Office reports to the Office of the Vice President for Academic Affairs and Provost. Key faculty and staff members involved in Program Management are as follows:

Principal Investigator and Director:	Lawrence C. Mohr, Jr., M.D.
Associate Director for Program Development:	John B. Dunbar, Dr. P.H.
Associate Director for Administration and Finance:	Gail C. Brubaker, B.S.
Co-Principal Investigator, Environmental Toxicology:	David Jollow, Ph.D.
Co-Principal Investigator, Environmental Epidemiology and Risk Assessment:	David G. Hoel, Ph.D.
Fiscal Analyst:	Anita G. Jefferson, B.S.
Administrative Coordinator:	Jill Canaday
Administrative Specialist:	Percilla E. Coaxum

3.0 Scientific Research

3.1 Environmental Toxicology Research Projects

3.1.1 Characterization of Species Differences in Trichloroethylene – Induced Peroxisome Proliferation and Hepatocyte Replication

Project Director: JoEllyn M. McMillan, Ph.D.

Executive Summary

The hepatocarcinogenicity of trichloroethylene (TCE) is thought to be related to the ability of its metabolites, trichloroacetic acid (TCA) and dichloroacetic acid (DCA), to induce peroxisome proliferative and/or hepatocyte mitogenesis in B6C3F1 mice and rats. Humans are considered to be less sensitive to TCE, but their susceptibility to peroxisome proliferation and hepatocyte mitogenesis is largely unknown. The relative susceptibility of human *vs.* B6C3F1 mouse hepatocytes to peroxisome proliferation is of key importance for the use of mechanistic information in the reassessment of the carcinogenic risk posed by environmental TCE. Of importance, the role of the peroxisome proliferator activated receptor a (PPARa) in the mitogenic response is unknown. It is believed that differences in the levels or activity of PPARa between humans and rodents is important in the relative insensitivity of human hepatocytes to traditional peroxisome proliferators. Thus, defining the role of PPARa in the mitogenic response and delineating differences in PPARa activity in humans vs. rodents would contribute key mechanistic information for assessing the hepatocarcinogenic risk posed to humans by TCE exposure. The overall goal of this proposal is two fold: (1) to enhance our understanding of the epigenetic basis for TCE-induced hepatocarcinogenicity; and (2) to improve the assessment of relative risk of human *vs.* the B6C3F1 mouse hepatocarcinogenicity.

Relevance

The ability of peroxisome proliferators to induce peroxisomal and non-peroxisomal enzymes, the mitogenic activity of these compounds and their hepatocarcinogenic potential varies among species and is dependent upon the particular chemical agent being used. The proposed studies will provide valuable mechanistic data for determining the relevance of the B6C3F1 mouse model for assessing the hepatocarcinogenic potential in humans of TCE and other peroxisome proliferators. The studies will provide a quantitative comparison of the relative responsiveness of human versus mouse and rat hepatocytes to peroxisome-proliferator-induced changes in activities and levels of key proteins and mRNAs.

Objective

The hepatocarcinogenicity of TCE is believed to be related to the ability of its metabolites, TCA and DCA, to induce peroxisome proliferative and mitogenic activity in B6C3F1 mice and rats. Humans are considered to be less sensitive, but

their susceptibility to peroxisome proliferation and mitogenesis is largely unknown. The role of PPARa in peroxisomal enzyme induction in rodents is well documented. However its regulation of other non-peroxisomal genes is less understood. Differences in the levels and activity of this transcription factor have been observed between human and rodent liver. Thus determining the role of PPARa activation in both the peroxisomal and mitogenic responses in human and rodent hepatocytes is important in assessing the relative hepatocarcinogenic risk to humans of TCE exposure. To this end our specific aims are as follows.

Specific Aim 1. To develop sensitive and selective approaches to measure the peroxisome proliferative and mitogenic responses in cultured liver cells

Specific Aim 2. To elucidate the mechanism for the short-term *in vivo* hepatocyte replication response

Specific Aim 3. To determine the involvement of the peroxisome proliferator activated receptor a (PPARa) in peroxisomal and cell replicative events in rodent and human hepatocytes.

Quarterly Accomplishments

Manuscript in press:

Walgren, JL, Kurtz, DT and McMillan JM. Lack of direct mitogenic activity of dichloroacetate and trichloroacetate in cultured rat hepatocytes. 2005. *Toxicology,* in press.

Poster presented:

JoEllyn M. McMillan and Keashia McKelvey. Protective Effect of Dibromoacetate in Primary Hepatocyte Cultures. Presented at the 2005 Society of Toxicology Meeting, New Orleans, March, 2005.

Performance Schedule and Status of Aims

We will continue to analyze, for hepatocyte replication determination, livers from PPARα knockout mice treated with TCE, TCA, DCA and Wy-14,643.

We are continuing studies to determine the anti-apoptotic effect of dihalogenated acetates, including dichloroacetate, on hepatocytes from $B6C3F_1$ mouse, rat and human liver.

3.1.2 Effects of Trichloroethylene Metabolites on Hepatic Cell-Cycle Regulatory Proteins and Transcription Factors

Project Director: David T. Kurtz, Ph.D.

Executive Summary

This project explores the hypothesis that the epigenetic carcinogenicity of TCE results from the mitogenic activity of its metabolites. Mitogenesis may occur either via the peroxisomal response or by an independent mechanism. There are two specific research objectives: to determine how TCE metabolites cause increased cell growth and division in the liver and to develop quantitative tools to allow direct comparison of the responsiveness of humans vs. the laboratory rodent. The experimental approach will utilize cultured hepatocytes the B6C3F1 mouse, Long Evans and Sprague-Dawley rats, and long-term cultures of human hepatocytes, which have retained their differentiated properties. The ability of TCE and/or its metabolites to induce: cdk mRNAs and proteins; cyclin mRNAs and proteins; CKI mRNAs and proteins; and cyclin/cdk activity will be assessed. The activation of transcription factors associated with cell division (AP1, NF kappaB, E2F) and the inactivation of transcription factors associated with the suppression of cell division (C/EBP) will also be determined. To determine the importance of the peroxisome proliferator activated receptor (PPAR) in these inductions, the studies will also be carried out on hepatocytes from PPAR alpha -/- ("knockout") mice. These studies will provide valuable insight into the molecular basis of the non-genotoxic carcinogenic effects of TCE and related hazardous compounds. Furthermore, the measurements of cell cycle regulatory protein activity, and of transcription factors associated with cell proliferation, may prove to be an accurate biomarker for hepatocarcinogenesis.

Relevance

Trichloroethylene is a widespread contaminant at DOE sites. The toxicity of this compound to humans continues to be controversial. The studies outlined above should provide specific evidence for or against the hepatotoxicity of TCE.

Objective

The scientific problem being addressed in this proposal is the molecular basis for the hepatocarcinogenicity of TCE metabolites. The general approach will be a combination of biochemical, molecular biological, and cell biological techniques. To this end our specific aims are as follows.

Specific Aim 1. To determine the molecular mechanism(s) by which TCE metabolites can serve as priming agents for mitogenesis in rodent hepatocytes and to determine if this effect can occur in human hepatocytes.

Specific Aim 2. To identify the effects of TCE metabolites on signal transduction cascades which may affect cell division in hepatocytes

Specific Aim 3. To determine the effects of TCE metabolites on the activity of hepatocyte transcription factors which regulate cell division, and whether these effects require PPAR.

Quarterly Accomplishments

1. Western blot analysis showed that DCA treatment of PPAR alpha knockout mice led to the induction of cyclin D1 and PCNA liver , and the transient suppression of hepatic C/EBP alpha, which normally serves to suppress cell division. This indicates that the transient mitogenesis in liver induced by DCA does not require the PPAR alpha receptor

2. We have developed a cell culture selection method by which isolated rodent and human hepatocytes can be immortalized while retaining their liver-specific properties

3. We have found that short term DCA treatment of hepatocytes does not induce phosphorylation of STAT3, although in vivo treatment of mice with DCA results in the induction of STAT transcription factors

Performance Schedule and Status of Aims

The project is on schedule and no significant changes in the specific aims are anticipated.

3.1.3 Human and Rodent Renal Proximal Tubular Cells as Model Systems to Study the Toxicity and Elimination of Trichloroethylene Metabolites

Project Director: Douglas Sweet, Ph.D.

Executive Summary

A growing body of evidence suggests that trichloroethylene (TCE) exposure induces hepatocellular carcinoma, nephrotoxicity, and nephrocarcinogenicity in rats. Further research has indicated that it is not the parent compound, TCE, but rather several of its metabolites, including trichloroacetic acid (TCA), dichloroacetic acid (DCA), and 1,2-dichlorovinyl-L-cysteine (DCVC) that are the causative agents of the associated renal and hepatic toxicities. The kidney and liver are target organs because they actively remove organic anions from the circulation and are therefore subject to high levels of accumulation of these negatively charged metabolites. In the case of TCE, metabolite formation, particularly at drinking water levels, is extremely rapid and target tissue exposure is largely determined by the relative rates of formation of the metabolites in the liver and their removal from the body by the renal proximal tubule cells (RPTCs). In other words, the peak metabolite concentrations and duration of exposure to the toxins are mostly determined by the kidney's ability to actively remove these substances from

the body. Therefore, the mechanisms governing the absorption, distribution, and excretion of these compounds likely play a central role in their associated toxicities.

Two gene families expressed in the kidney and liver that mediate the transport of small organic anions are the Organic Anion Transporter (OAT) family and the Organic Anion Transporting Polypeptide (Oatp) family. Recently, DCVC uptake by rabbit proximal tubules was demonstrated to be blocked by *p*-aminohippurate (PAH), the prototypical substrate for OATs. This finding suggests the OAT family of transporters is involved in the absorption, distribution, and excretion of TCE metabolites (Oatps do not transport PAH). In further support of this, DCVC inhibited organic anion transport mediated by the rabbit and human orthologs of organic anion transporter 1 (Oat1) expressed in heterologous cell systems.

Variation in the renal elimination rate constants of the ultimate carcinogenic metabolites, TCA and/or DCA, is likely to be a crucial factor in physiology based pharmacokinetic (PBPK) modeling for sensitive populations and in risk assessment based upon such modeling. Current PBPK models used in the assessment of toxicological endpoints for TCE exposure do not incorporate the contribution of active transport mechanisms that impact body compartment distribution and concentration levels, including organ-specific accumulation and/or secretion of TCE and its metabolites. Therefore, the proposed research will quantify the contribution of active transport to the absorption and secretion of the acidic metabolites of TCE (TCA and DCA), as well as the metabolite DCVC, to increase the complexity and accuracy of PBPK models for TCE exposure.

Relevance

It is well known that TCA has a long plasma half-life in rodents and humans and that this is a major contributor to the high "area under the curve" (AUC) for TCA. Restrictive binding to plasma albumen has been suggested as the molecular basis for this long half-life. However, recent studies have indicated that the extent of binding is less than 90% and hence should be non-restrictive for glomerular filtration. An alternative explanation for the lack of rapid renal excretion is glomerular filtration followed by reabsorption from the lumen of the nephron by an active transport process. Thus, renal organic anion transporters may play a significant role in determining the AUC for TCA, and hence be a prime determinant in the dose/response relationship for hepatocellular carcinoma. Knowledge of the rate constants characterizing renal transport would be helpful in validation of PBPK models of TCE hepatocellular carcinoma and essential in the identification of individuals at highest risk.

Knowing which transporters are responsible for mediating the elimination and/or reabsorption of DCVC, TCA, and DCA would aid in the prediction and modeling of potential drug-xenobiotic interactions that might exacerbate or alleviate the toxic effects of these compounds. Understanding the molecular basis for differing sensitivities of exposure between rodents and humans would aid the process of extrapolating rodent bioassay data to humans. This information might also make it possible to include toxicogenetic parameters in risk assessment models to identify individuals/populations

predisposed to increased risk of nephrotoxicity and hepatocarcinogenicity after TCE exposure. Expression levels of the identified transporters may even serve as useful biomarkers for the identification of such individuals/populations.

Objective

Preliminary investigations will determine the ability of unlabeled TCA, DCA, and DCVC to inhibit the function of cloned organic anion transporters expressed in *Xenopus* oocytes and transfected cultured mammalian cell lines. Once potential transporters have been identified, their ability to mediate transport of radiolabeled TCA, DCA, and DCVC will be directly examined and the kinetic parameters of transport determined. These observations will then be confirmed in intact tissue systems (*i.e.,* kidney and liver slices) and isolated primary RPTC from mouse, rat, rabbit, and humans. The impact that interindividual variation in transporter expression and function has on susceptibility to TCE induced renal and hepatic toxicity will also be examined.

In order to establish the role active transport of TCA, DCA, and DCVC plays in their associated nephrotoxicity/hepatocarcinogenicity the following specific objectives will be addressed:

Specific Aim 1. The identification of specific transporters controlling the systemic disposition of DCVC, TCA, and DCA in mouse, rat, rabbit, and human RPTC.

Specific Aim 2. Characterization of mouse, rat, rabbit, and human transporter kinetic constants for DCVC, TCA, and DCA.

Specific Aim 3. Assessment of potential drug-xenobiotic interactions for the mouse, rat, rabbit, and human transporter orthologs and their influence on the toxicity of DCVC, TCA, and DCA.

Specific Aim 4. Incorporation of this information into PBPK models of TCA, DCA, and DCVC distribution for risk assessment purposes.

Quarterly Accomplishments

1. We are continuing preliminary functional characterization of apical organic anion transport in confluent mouse RPTC monolayers. Inhibitor-sensitive uptake of the organic anion transport system substrates *para*-aminohippurate and estrone sulfate is observed. Furthermore, phloridzin-sensitive alpha-methylglucopyranoside uptake, considered the hallmark test for Na^+-dependent glucose transport in RPTCs, is also observed. These results indicate that the isolated primary cells represent a population enriched for RPTCs and that the cultured monolayers are exhibiting characteristics consistent with that of the intact polarized renal epithelium.

2. We are examining the use of a histochemical assay for alkaline phosphatase activity. High alkaline phosphatase activity correlates with RPTCs.

3. We are continuing to examine directly the ability of TCA, DCA, and DCVC to act as inhibitors of apical renal organic anion transporters expressed in isolation using *Xenopus* oocyte expression assay.

4. We are expanding our survey of gene expression in the primary RPTC monolayers by polymerase chain reaction (PCR) to include some cortical collecting duct specific genes. Low to no expression of these markers will further establish that the primary cell cultures are enriched for proximal tubule cells and that they represent a valid *in vitro* model system.

Performance Schedule and Status of Aims

Neither the performance status nor the status of aims has changed.

3.1.4 Effect of Genetic Variation and of Ethanol on the Formation of Trichloroacetic Acid, a Putative Hepatocarcinogenic Metabolite of TCE

Project Director: David McMillan, Ph.D.

Executive Summary

During this third quarter we are continuing to collect and perform studies on chloral hydrate metabolism using human hepatocyte cultures. We have begun to observe some variability in the formation of TCA and TCE-OH, though more human samples will be required to determine the extent of the variability and the relationship to genotype. We are in the process of determining how many samples it will take to determine variability using power calculations using the known variability in the metabolism of ethanol. We also continue to detect the formation of DCA in human samples, and it appears that formation of this metabolite is real and not an artifact. We plan confirm its identity using GC/MS analysis in the next quarter. An abstract to the Society of Toxicology meeting on these data have been prepared.

Relevance

The utility of PBPK modeling of blood TCA levels as a dose metric for liver exposure to TCA after TCE ingestion is well accepted. Unfortunately, the relationship between TCE exposure and liver levels (AUC and peak concentrations [which may vary independently]) are complex and are very likely to show major differences among human sub-populations. These differences may underlie enhanced susceptibility (or resistance) by both genetic and environmental factors. The interaction of the genetic and environmental factors may further alter the relationship between applied dose of TCE and liver exposure to TCA. The proposed studies will be used in collaboration with projects 5

and 6 to improve the reliability and applicability of PBPK modeling in the assessment of risk of humans to TCE.

Objectives

1. To determine the kinetic constants for conversion of CH to TCA and TCOH in hepatocytes from the target species, mice and rats (including the back reaction of TCOH to CH and TCA).
2. To determine the kinetic constants for conversion of CH to TCA and TCOH in human hepatocytes.
3. To characterize the isoform composition of human hepatocytes by enzymic and DNA array technology.
4. To determine the effect of ethanol on the redox state of hepatocytes from mice, rats and humans.
5. To determine the effect of ethanol on conversion of CH to TCA and TCOH in hepatocytes from mice, rats and humans.

Specific Aim 1. To determine the kinetic constants for conversion of CH to TCA and TCOH in hepatocytes from the target species, mice and rats (including the back reaction of TCOH to CH and TCA).

Specific Aim 2. To determine the kinetic constants for conversion of CH to TCA and TCOH in human hepatocytes.

Specific Aim 3. To characterize the isoform composition of human hepatocytes by enzymic and DNA array technology.

Specific Aim 4. To determine the effect of ethanol on the redox state of hepatocytes from mice, rats and humans.
Specific Aim 5. To determine the effect of ethanol on conversion of CH to TCA and TCOH in hepatocytes from mice, rats and humans.

Quarterly Accomplishments

During this third quarter we are conducting experiments with B6C3F1 mouse livers to determine the factors that may contribute to the variability that we have observed in the human hepatocytes studies. Experiments are being performed to establish the reliability, reproducibility and the limit of detection of the GC assay for chloral hydrate metabolism. In addition, we are conducting experiments to learn which experimental model (i.e., liver homogenate, cryopreserved hepatocytes, or freshly isolated hepatocytes) is the best for determining human variability. We have also begun to determine ADH geneotype in the human livers that we presently have. Preliminary power calculations performed by our graduate student on the project, Apryl Bronley-DeLancey, indicate that number of human livers that will be needed to assess the extremes of variability (based on literature values for the metabolism of ethanol) is within reach of our capability to acquire these livers

from commercial sources. When we have a better idea about what other factors contribute to the variability in mouse liver metabolism, we will return to the human liver studies.

Performance Schedule and Status of Aims

The project is on schedule and no significant changes in aims have occurred.

3.1.5 Presystemic Elimination of Trichloroethylene and its Interactions with Alcohol: How Important are They at Environmental Exposure Levels?

Project Director: James V. Bruckner, Ph.D.

Executive Summary

Although extremely high doses of trichloroethylene (TCE) are required to produce tumors in mice and rats, there is concern on the part of the EPA and others that even trace (i.e., environmental) levels may present a cancer risk to humans. The human body has a number of processes to protect against such low level toxic insults, including first-pass, or presystemic elimination. Volatile organic chemicals (VOCs) such as TCE that are absorbed from the gut are subject to metabolism by the liver and exhalation by the lungs, before they reach the arterial circulation and are distributed systemically. It has been theorized, but not demonstrated experimentally, that all of low oral doses of VOCs are removed by presystemic elimination. It will be necessary to develop very sensitive analytical techniques in order to conduct experiments with environmentally-relevant levels of TCE. Demonstration [experimentally and by physiologically-based pharmacokinetic (PBPK) modeling], that all of low oral doses of TCE are eliminated, would have a profound effect on extrahepatic cancer and non-cancer risk assessments of TCE.

Alcohol (i.e., ethanol) and a number of other compounds are known to stimulate formation of increased amounts of cytochrome P450 2E1 (CYP2E1) in the liver. CYP2E1 is the key enzyme that initiates the oxidation of low doses of TCE to potentially mutagenic metabolites. Thus it is reasoned that drinkers metabolically activate a greater percentage of their systemically-absorbed dose of TCE to carcinogenic metabolites. Similarly, populations with genetically-determined elevations of CYP2E1 might also be anticipated to be at increased risk. The EPA uses this reasoning in their most recent health risk assessment of TCE, to support their choice of the most conservative (i.e., linear, no-threshold) mathematical model to predict cancer risks. Preliminary PBPK modeling efforts suggest that elevated CYP2E1 activity will not result increased metabolism of low, environmentally-relevant doses of TCE. Every human has CYP2E1 activity far in excess of that necessary to metabolize all of low doses. Since all of trace amounts of TCE are metabolized, it is reasonable to conclude that increased metabolic capacity due to alcohol, drugs, genetics, etc. is inconsequential. Laboratory experiments and PBPK modeling will be carried out to prove this hypothesis.

Relevance

As described above, this research project is directly relevant to current and proposed EPA regulatory standards for drinking water contamination by TCE. The EPA concludes, through both its cancer and non-cancer risk assessments (EPA, 2001), that exposure to even minute levels of TCE is associated with low-level human risks. It is concluded that certain subpopulations with genetically- or drug-induced elevations of P4502E1 (the enzyme responsible for formation of toxic metabolites of TCE) will be at significant risk. Preliminary research with other well-metabolized chemicals indicates that this is not true. The proposed research with alcohol should definitively establish this for TCE. The second low-dose phenomenon to be investigated here will be presystemic, or first-pass elimination. The liver and lungs act in concert to eliminate ingested VOCs before they reach the systemic/arterial circulation. It is postulated that virtually all of trace levels of TCE in drinking water are removed, before they reach and present a hazard to extrahepatic target organs such as the lungs and kidneys. Experiments have been designed and a PBPK model will be developed in collaboration with Dr. Fisher to characterize the capacity of this protective mechanism under different TCE exposure conditions.

Objectives

1. Develop and validate assays of TCE and its major metabolites in biological samples, including blood, tissues and urine. The assays should be sufficiently sensitive to utilize in animal experiments employing very low doses of TCE.

2. Accurately determine the capacity and dose-dependency of presystemic elimination of orally-administered TCE. Characterize the influence of dose and dosage regimen on the systemic disposition/effects of TCE and related VOCs.

3. Establish the influence (or lack thereof) of ethanol on the metabolic activation of low oral doses of TCE. Determine whether the ratio of the metabolites trichloroacetic acid (potentially carcinogenic) and trichloroethanol (non-carcinogenic) is altered by ethanol.

Specific Aim 1. To determine the capacity and dose-dependency of presystemic elimination of ingested TCE and to delineate the relative contribution of the liver and lungs.

Specific Aim 2. To establish the influence (or lack thereof) of ethanol on the metabolic activation of environmentally-encountered doses of TCE.

Specific Aim 3. To determine whether the ratio of the metabolites trichloroacetic acid (TCA) (potentially carcinogenic) and trichloroethanol (TCOH) is altered by co-ingestion of ethanol.

Quarterly Accomplishments

1. Our investigation of the influence of cytochrome P450 2E1 (CYP2E1) induction on trichloroethylene (TCE) metabolism and pharmacokinetics is ongoing. The rationale and experimental approach for this work were described in the last quarterly report. The objective is to prove or disprove the Hypothesis that: "Drug-induced and genetically-determined elevations in CYP2E1 are inconsequential to TCE cancer risks, because CYP2E1 activity in normal individuals is far in excess of that needed to metabolize/metabolically activate all of trace amounts of the chemical. Thus, further increases in CYP2E1 activity are of no consequence."

2. We have completed a series of experiments designed to assess the influence of pyridazine (PZ), a potent CYP2E1 inducer, on the metabolism of a series of doses of TCE. Results of the assessment of one dose (i.e., 200 mg/kg) were reported in my last quarterly report. In each instance, some groups of male Sprague-Dawley rats were pretreated for 3 days with 200 mg PZ/kg ip. The pretreated and naive groups of rats were then dosed orally with 10, 50 or 200 mg TCE/kg. Serial micro blood samples were taken from each animal via an indwelling carotid artery cannula for up to 48 hours post dosing and analyzed by headspace gas chromatography for their TCE, chloral hydrate (CH), trichloroethanol (TCOH), dichloroacetic acid (DCA) and trichloroacetic acid (TCA) content.

3. The influence of PZ/CYP2E1 induction on TCE metabolism was dose-dependent. Systemic elimination of TCE at the highest (200 mg/kg) dose was markedly enhanced, but TCE elimination at the lowest (10 mg/kg) dose was unaffected. PZ resulted in a substantial decrease in TCA levels and an increase in TCOH levels, as reflected by the areas under the blood concentration versus time curves (AUCs) for each metabolite. This shift in metabolite profiles was most pronounced at 200 mg/kg, and barely manifest (i.e., significantly different from naive animals) at 10 mg/kg. PZ had minimal effects on the toxicokinetics (including elimination half-life) of TCOH, as evidenced by plasma concentration time-profiles in rats administered TCOH iv. The increases in TCOH AUCs resulted primarily from increased TCOH formation from TCE. In direct contrast, the toxicokinetics of TCA was significantly altered by PZ. TCA AUCs and half-lives were consistently lower in induced rats. This was true both for TCA formed from TCE and for TCA in rats given the metabolite iv. The data from the iv experiment showed that increased TCA clearance was responsible for the lower AUCs and shorter half-lives.

4. At present, the EPA is largely focused on TCA as a proximate liver carcinogen. Thus, lower circulating TCA levels in CYP2E1-induced animals would logically be associated with reduced cancer risk. It would be of real interest to determine whether the same phenomenon is produced by other CYP2E1 inducers (e.g., ethanol, acetone, acetaminophen). Our demonstration of the dose-dependency of effects of PZ-CYP2E1 induction on TCE metabolism may also have important

practical implications. Relatively little effect was seen on the 10 mg/kg dose of TCE. This finding is consistent with our Hypothesis in the first paragraph. The sensitive analytical methods we have developed will make it possible to test the Hypothesis at lower, more environmentally-relevant TCE exposure levels.

5. Our findings were presented at the annual national meeting of the Society of Toxicology this March, in New Orleans. The references to the three abstracts are:

   Lee, S., Muralidhara, S., Anand, S.S., White, C.A. and Bruckner, J.V.: Cytochrome P450 2E1 (CYP2E1) induction by pyridazine produces qualitative and quantitative changes in the metabolism of trichloroethylene to potentially carcinogenic metabolites. *Toxicologist* 84: 149 (2005).

   Hines, C., Muralidhara, S., White, C. and Bruckner, J.V.: The effect of dose on the metabolic profile of trichloroethylene. *Toxicologist* 84: 255 (2005).

   Keys, D.A., Lumpkin, M.H., Bruckner, J.V. and Fisher, J.W.: Incorporation of trichloroacetic acid plasma binding in human and mouse in trichloroethylene risk assessment. *Toxicologist* 84: 81 (2005).

6. One paper was accepted for publication:

   Delinsky, A.D., Bruckner, J.V. and Bartlett, M.G.: A review of analytical methods or the determination of trichloroethylene and its major metabolites chloral hydrate, trichloroacetic acid, and dichloroacetic acid. *Biomed. Chromatogr.* in press (2005).

7. One manuscript was submitted for publication:

   Lumpkin, M.H., White, C.A., Muralidhara, S., Fisher, J.W., Dallas, C.E. and Bruckner, J.V.: Dose-, species-, and time-dependency of blood and tissue trichloracetic acid levels as dosimeters of trichloroethylene exposure. Submitted to *Toxicol. Sci.* (2005).

Performance Schedule and Status of Aims

Neither the performance status nor the status of aims has changed.

3.1.6 PBPK Modeling of Toxic Metabolites of Trichloroethylene in Rats, Mice and Humans: Predicting the Health Risks Posed by Low Level Exposure to TCE

Project Director: Jeffery W. Fisher, Ph.D.

Executive Summary

Trichloroethylene (TCE) remains one of the most common ground water contaminants found in the US because of its disposal and use practices by the private sector, DOE and DOD. The projected costs for remediation of TCE in the federal sector is well over $1 B. The health risks of TCE were recently reviewed by several scientists and published as a monologue in an Environmental Health Perspectives (EHP) Supplement (Vol. 108(2), 2000). Since the EHP publication on TCE, the US EPA released a draft 'regulatory risk assessment for TCE' to the authors of the EHP monologue and asked the authors to comment on their document. In July 2002 the US EPA convened a scientific review panel to review their most recent draft TCE document. Physiologically based pharmacokinetic (PBPK) models were used as an aid in dose-response assessment (risk assessment) for cancer and non-cancer toxicological endpoints. Five PBPK models were used on various human and rodents studies for cancer and non-cancer endpoints. Several data gaps were identified as the US EPA attempted to use the PBPK models of Fisher, Clewell and Barton. In some cases the PBPK models were inappropriately or insufficiently exercised. The objective of this project is to develop a single robust PBPK model for TCE for rodents and humans by incorporating new metabolic and kinetic data published since 1999, and by conducting limited critical metabolic and pharmacokinetic experiments in rodents to fill data gaps. The refined PBPK model for TCE and metabolites in laboratory animals and humans will be exercised in an appropriate manner, and the results will be used to reduce the uncertainties associated with assessing the human health risks posed by low-level environmental exposure to TCE.

Much progress has been achieved over the last 5 years in understanding the quantitative aspects of metabolism of TCE in humans and rodents and in understanding the toxic and carcinogenic potential of the acid metabolites that are formed from metabolism of TCE. PBPK models have progressed from models that simply describing the parent chemical to PBPK models that contain sub models describing the formation and kinetics of metabolites such as trichloroacetic acid (TCA), trichloroethanol, chloral hydrate and in some cases, dichloroacetic acid. Colleagues of mine and I have developed and published most of the PBPK models for TCE and metabolites in humans and rodents with financial support from the USAF, US EPA and Strategic Environmental Research and Development Program (SERDP). The US EPA used early-unpublished versions of our most recent PBPK models for mice and humans in their current draft risk assessment document.

Relevance

The scientific issues related to determining the health risks posed by low levels of TCE in the environment are relevant to many other solvents found in water supplies. If sound science and extrapolation methodology can be demonstrated for this chemical, then other chemicals can be evaluated in a similar manner. This could lead to a potential saving of multiple millions of dollars in unnecessary clean-up costs.

Objectives

1. Harmonize current PBPK models used by the US EPA into one PBPK model for TCE and metabolites. Incorporate newly published and unpublished data in humans and rodents. New data sets include published and unpublished rat data on first pass metabolism of TCE from the laboratory of Dr. Jim Bruckner at the University of Georgia, published human and unpublished rat data on glutathione conjugation of TCE [(S-(1,2-Dichlorovinyl) Glutathione (DCVG)] obtained by Dr. Larry Lash at Wayne State University, and published Epidemiology studies performed in Europe, where urinary excretion of TCA was quantified.

2. Conduct laboratory studies to refine PBPK model predicted dose metrics in laboratory animal and humans that will be used in the formulation of the final product of this project, namely a TCE human health risk assessment. Determine the stoichiometric yield of DCVG for relevant doses of TCE in rats. Information on DCVG will provide data to develop the DCVG pathway in a PBPK model for TCE and to offer plausible dose-metrics that can be associated with the risk of kidney cancer in humans. Colleagues and I have time course data for DCVG in humans exposed to TCE vapors [Lash, LH, DA Putt, WT Brashear, R Abbas, J Parker and JW Fisher. 1999. Identification of S-(1,2-Dichlorovinyl) Glutathione in the Blood of Human Volunteers Exposed to Trichloroethylene. *J. Toxicol. Environ. Health* Part A, 56, 1-21].

3. Conduct laboratory studies to evaluate how much dichloroacetic acid (DCA) is formed metabolically from TCE. This minor metabolite remains an important risk assessment issue because of its carcinogenic potency and the requirement that the US EPA account for cumulative risks. DCA is the number one by-product from chlorination of water. Thus, to account for the health risks poised by TCE in drinking water, the health risks from exposure to DCA itself must be quantified and accounted for in the health risk assessment of TCE.

4. Perform a cancer and non-cancer risk assessment for TCE using the harmonized single PBPK model for TCE and metabolites. The risk assessment will rely on ‘mode of action’ hypotheses and theoretical assumptions for low dose extrapolations. Relevant human data sets will be incorporated into the analyses.

Specific Aim 1. To harmonize current PBPK models used by the US EPA into one PBPK model for TCE and metabolites by incorporating newly published and unpublished data in humans and rodents.

Specific Aim 2. To examine the metabolism of TCE in rodents with emphasis on the dose-dependence of conversion of TCE to DCVC.
Specific Aim 3. To re-examine the dose-dependence of conversion of TCE to DCA in laboratory animals.

Specific Aim 4. To perform a cancer and non-cancer risk assessment for TCE using the harmonized single PBPK model for TCE and metabolites.

Quarterly Accomplishments

1. Animal studies are ongoing to determine the fractional yield of metabolically formed dichloroacetic acid (DCA) from metabolism of TCE in mice and rats.

2. The development of a human PBPK model for dichloroacetic acid (DCA) has just started with a Ph.D. student, Ting Li.

Performance Schedule and Status of Aims

No research on the DCVC pathway is scheduled in the near future (Specific aim-2). Neither the performance status nor the status of other aims has changed.

3.1.7 Trichloroethylene Exposure and Host Genetic Factors in Autoimmune Diseases

Project Director: Janardan P. Pandey, Ph.D.

Executive Summary

Several environmental chemicals have been associated with autoimmune diseases; however, in most cases, a definitive role for environmental agents in the initiation or exacerbation of autoimmune diseases is not firmly established. In particular, very little is known about the effects of the host genetic factors on the ability of environmental agents to initiate, perpetuate, or prevent autoimmune diseases. Identification of disease-associated single nucleotide polymorphisms (SNPs) will aid in fine-mapping the disease susceptibility genes. Moreover, the elucidation of the genomic response to environmental toxicants— toxicogenomics—may be helpful in identifying individuals with increased susceptibility to environmental agents. Understanding the role of environmental chemicals and the genetic factors in the induction of autoimmune diseases will aid in designing new tools for diagnosis and prophylaxis of these diseases. In addition to the possible identification of genes for systemic sclerosis (SSc), systemic lupus erythematosus (SLE), and myositis, the proposed investigation will contribute to the construction of haplotype maps of SNPs on chromosomes 2,6,10, and 14 that may be used for studies involving other diseases whose causative genes are known to be on these

chromosomes. As emphasized at a recent NIH-sponsored meeting, building haplotype maps is the next phase of the human genome project.

Relevance

Understanding the role of chemicals like TCE and the host genetic factors in the induction of autoimmune diseases will be helpful in designing new tools for diagnosis and prophylaxis of these diseases. Identification of the disease-associated genetic markers may shed further light on the role of these polymorphic genetic systems in autoimmunity.

Objective

The overall long-term goal of this project is to identify the genetic and environmental factors which contribute to the pathways to autoimmunity. In particular, we would like to determine how certain genes of the immune system and those involved in the bioactivation of particular environmental toxicants interact in causing autoimmune diseases. We also plan to develop a murine model for use in dissecting the biological mechanisms underlying environmentally associated autoimmunity. Specifically, we would like to determine whether the exposure of mice to TCE causes activation of microchimeric cells and the appearance of dermal inflammation and fibrosis similar to that of graft-versus-host disease, a condition with remarkable similarities to SSc. To the above end, during this cooperative agreement period, using a case-control study design, the proposed study will address the following specific aims:

Specific Aim 1. (a) To further estimate the magnitude of the association between TCE/silica exposure and SSc, SLE, and myositis and (b) to determine if the effect is modified by the prevalence of disease-specific autoantibodies — anti-topoisomerase I, anticentromere, and anti-RNA polymerase I and III in SSc; anti-Sm in patients with SLE; and anti-tRNA synthetases in myositis.

Specific Aim 2. To compare the distribution of particular genetic markers (HLA, TNF-a, TNF-b, IL-1b, IL-1RA, IL-10, CTLA-4, DNASE1, cytochrome P450IIE1, GM, and KM) and the recently-identified SNPs closely linked to them, among TCE/silica exposed SSc, SLE, and myositis patients with (a) non-exposed patients and (b) non-autoimmune controls.

Specific Aim 3. To compare the association of autoantibodies with the immunogenetic markers among TCE/silica-exposed and nonexposed SSc, SLE, and myositis patients.

Specific Aim 4. To develop a murine model for use in examining the role of microchimeric cells and TCE exposure in SSc pathogenesis.

Specific Aim 5. To construct transgenic mice with different combinations of CTLA-4 genotypes and expose them to TCE to determine the possible interactive effects of CTLA-4 alleles and TCE exposure in producing dermal inflammation and fibrosis.

Quarterly Accomplishments

1. The following manuscript has been accepted for publication:

   Hudson LL, Rocca KM, Kuwana M, Pandey JP. Interleukin-10 genotypes are associated with systemic sclerosis and influence disease-associated autoimmune responses. *Genes Immun* 2005; in press.

2. Results of our most recent analysis in the Carolina Lupus Study show that particular CTLA-4 genotypes at position –1661 are associated with the prevalence of anti-Sm ($p = 0.01$) and anti-SSB antibodies ($p = 0.0005$). Anti-Sm antibodies are found mainly in SLE patients, whereas anti-SSB antibodies are present in SLE patients and in patients with Sjögren's syndrome. A manuscript describing these findings is in preparation.

3. Analysis of CTLA-4 and IL-10 genotypes in the Korean patients with SLE showed that particular IL-10 genotypes at –2763 are associated with the prevalence of anti-Sm antibodies. Furthermore, certain genotypes at the two loci interact to increase the risk of acquring SLE (OR = 2.69, 95% CI: 1.70-4.26).

4. The following manuscript, which describes our most recent findings concerning molecular mimicry in scleroderma, has been submitted for publication:

   Namboodiri AM, Rocca KM, Kuwana M, Pandey JP. Association of IgG antibodies to human cytomegalovirus protein UL83 with systemic sclerosis. *J Med Virol* 2005; submitted.

5. Our initial series of experiments on murine microchimerism and TCE exposure are near completion. Fluorescent *in situ* hybridization to detect microchimeric (male) cells in female mice treated with TCE has not revealed an increased prevalence of these cells in mice treated with TCE, compared to controls. PCR assays to detect male DNA in female mice are in progress. Activated T cells—as determined by the presence of cells expressing the IL-2 receptor—were not detected in the skin of mice treated with TCE. Thus, TCE does not appear to induce pathology of the skin, kidney or spleen under the conditions used in this study. A murine model of SSc that involves graft-versus-host disease is being developed to delineate the mechanisms underlying TCE exposure-SSc association.

Performance Schedule and Status of Aims

Neither the performance schedule nor the status of aims has changed.

3.1.8 Immunological Effects of Trichloroethylene Exposure

Project Director: Gary S. Gilkeson, M.D.

Executive Summary

In previous periods of funding, we have evaluated immunological function after chronic exposure to TCE via drinking water in adult lupus-prone (NZB/NZW) and normal inbred strains of mice (B6C3F1). Furthermore, we have developed a polyclonal rabbit serum that binds to TCE/protein adducts. In this series of experiments, TCE accelerated the onset and severity of lupus-like disease in NZB/NZW mice. Significant increases in autoantibody production also developed in the B6C3F1 strain of mice, suggesting potential development of autoimmune effects even in 'normal' mice. We believe these data, when combined with data from other laboratories, indicate that TCE is an environmental inducer/accelerator of autoimmune disease. Based on recent concerns regarding chronic exposure of TCE to families living at or near Camp Lejune, NC, we propose to utilize our mouse models to evaluate the impact of TCE during early developmental periods of the immune system and assess predisposition or initiation of autoimmune disease. Thus, immune status should be evaluated after full life exposures to TCE to include stages of *in utero* development, neonatal development, puberty, and early adulthood. There is growing concern about the effects of *in utero* and childhood exposure to environmental toxins. It is of obvious importance to determine if *in utero* exposure to common environmental toxins has effects on the developing immune system. These experiments in normal and lupus-prone mice will provide insight into potential effects on immunological function and the development of autoimmune disease that can be used in assessment of risk for the human population, and in particular, children.

Relevance

There is growing concern about the effects of *in utero* exposure to environmental toxins. It is of obvious importance to determine if *in utero* exposure to common environmental toxins has effects on the developing immune system. These experiments in normal and lupus prone mice will provide insight into potential affects on the immune system that can then be assayed in humans exposed *in utero* to TCE. We will also hope to develop potential assays for TCE exposure using the now available anti-TCE adduct polyclonal sera.

Objective

The purpose of this project is to define the impact of TCE exposure on immunological function, with particular emphasis on autoimmune disease.

Specific Aim 1. Determine the immunological effects of *in utero* and early life exposure to low-levels of TCE (1,000 ppb and 10,000 ppb in the drinking water) in a non-autoimmune prone mouse strain (B6C3F1), with particular emphasis on the detection of autoimmune manifestations.

Specific Aim 2. Determine the effects on autoimmune disease development/progression in NZB/NZW mice exposed *in utero* and during early life to low-levels of TCE (1,000 ppb and 10,000 ppb in the drinking water). Effects attributed to *in utero* and early life exposure will compliment earlier studies with adult mice as the same strains of mice and levels of TCE will be utilized. Furthermore, this study will also permit direct comparisons between the immune effects of male and female mice exposed to TCE during these early developmental periods.

Quarterly Accomplishments:

1. The first study with MRL +/+ mice has been completed. They were treated with TCE in utero and then postnatally to approximately one year of age. Autoantibody production and proteinurea were assessed monthly beginning at 4 months of age. At study termination lymphocyte proliferation, immune organ cellularity, and lymphocyte immunophenotypes were assessed. Increased onset of autoantibodies was not detected in this model. Due to the length of time for untreated mice to develop disease symptoms it is concluded that this is not an appropriate model for assessing TCE-induced autoimmunity at low levels of exposure following gestational exposure. Therefore, NZB/NZW mice are being order to assess in utero exposure in a different strain. Additionally, we have begun to treated B6C3F1 mice again to confirm the results from the tumor challenge model completed in the spring of 2004.

Performance Schedule and Status of Aims:

No changes have been made in the status of aims.
Work on Specific Aim 2 has begun. The auto-immune prone mice have arrived, have been bred and have been dosed with TCE. The pups are currently being dosed with TCE. Autoantibody production assessments and urinary protein determinations began in the pups and are being assessed monthly.

3.1.9 Molecular Mechanism of Pathogenesis in a Model of Trichlorethylene-Induced Congenital Heart Disease: Roles of Growth Factors, Extracellular Matrix (ECM) Proteins, and Matrix Metalloproteinases (MMPs)

Project Director: Stanley Hoffman, Ph.D.

Executive Summary

Increased numbers of heart defects occur in children born where the water supply is contaminated with trichloroethylene (TCE), suggesting that TCE is teratogenic in humans. TCE has been reported to have teratogenic effects on chick and rat embryo hearts, without apparent effect on other organs. Heart malformations usually involve structures that form by epithelial-mesenchymal transformation (EMT). In this process which is repeated several times during heart development, a subset of cells in an epithelial sheet detach and migrate into the underlying basement membrane where they then differentiate in a novel direction. For example, endocardial cells undergo EMT and differentiate into valves and septa. In our laboratory, we use a cell line, QCE-6 cells, and explants of embryonic heart tissue to model EMT. This cell line has allowed us to identify biochemical changes that accompany and control EMT. Of particular interest are developmental changes in proteins present in the ECM and enzymes involved in the remodeling of the ECM that are capable of regulating cell behavior. Specifically, chondroitin sulfate proteoglycans may be critical components of the ECM because they are present in dynamically changing distributions in the developing heart and have been shown to regulate cell-cell and cell-ECM adhesion and subsequent intracellular signaling. MMPs are a major family of enzymes involved in the remodeling of the ECM. Moreover, our recent studies demonstrate that blocking MMP activity blocks both EMT and accompanying cell differentiation.

Relevance

The purpose of this project is to identify the molecular mechanisms associated with normal EMT and heart development that are affected by TCE. This information will provide a basis to assess the teratogenic potential of TCE for humans and, if TCE is indeed teratogenic in humans, to determine whether some individuals may show super-susceptibility. These studies may also suggest better methods to recognize and treat cardiac malformations induced by TCE.

Objectives

1. Determine whether TCE or its metabolites affect the morphology of the developing chicken and rodent hearts *in vivo*.

2. Determine when in development TCE-induced heart defects first appear and in what region of the heart.

3. Determine whether the morphological defects induced by TCE can be correlated with concomitant biochemical defects, particularly in components of the ECM involved in EMT including MMPs and chondroitin sulfate proteoglycans.

4. Determine whether experimentally reversing the effects of TCE on the composition and function of the ECM will also reverse its effects on heart morphology.

Quarterly Accomplishments

1. Concerted effort was placed on obtaining a large number of stage-paired whole embryo cultures treated with TCA (0, 10, 40 and 80 ppm). We now have approximately 80 specimens embedded in paraffin that are in the process of being sectioned for histochemistry and immunostaining (multiple fixatives for optimal antibody reactivity).

2. Testing was initiated for potential teratogenic effects of DCVC on endocardial morphogenesis. Preliminary results suggest a dose-dependent (0.5 to 5 ppm) loss of cell-cell adhesion of endocardial cells in collagen gel culture. However, there does not appear to be any obvious effect on cushion mesenchymal cells. Higher doses (50 ppm) of DCVC were overtly toxic to all cell types.

3. A pilot study was undertaken to determine if we could use MALDI MS to profile changes in the proteome of individual cushions due to embryo exposure to TCE and its metabolites. The results are very promising. We are currently able to obtain good spectra from one atrioventricular cushion from a stage HH 25 embryo. This is older than our target stage (HH18) so should we be unable to analyze individual HH18 hearts we may need to pool samples to achieve sufficient material for analysis. However, the availability of this powerful technology opens up new avenues for assessing TCE affects on heart morphogenesis.

Performance Schedule and Status of Aims:

In the coming quarter we will: i) assess histological and molecular consequences of TCA exposure on cushion morphogenesis, ii) continue in the characterization of DCVC effects on tissue culture explants to complement future whole embryo culture analysis, and iii) further explore the potential of using MALDI MS to profile phenotypic changes in heart development due to TCE exposure.

3.1.10 Immunotoxicological Assessment of Non-degraded and Biodegraded PCB Mixtures

Project Director: Lucille London, Ph.D.

Executive Summary

The long-term goal of our laboratory is to understand the biological process by which complex mixtures of contaminants can be degraded in the environment and to apply that knowledge to better understand potential human health effects associated with exposure. We will focus on the biodegradation of complex mixtures of polychlorinated biphenyls (PCBs) and their subsequent immunotoxicological effects. The potential for existing and newly emerging bioremediation technologies to treat complex waste sites is based upon their ability to remove these chemicals from contaminated environments. However, there has been little attempt to correlate disappearance of contaminated material with a discernible decrease in the health hazards associated with biotreated materials. Little is known about the immunotoxicity of the partial degradation products of PCBs; in particular whether the spectrum of effects may be different from that of the parent compounds. In addition, the molecular mechanism by which PCBs can effect the immune response is poorly understood. Biological effects of PCBs in animals have mostly been attributed to coplanar congeners, although effects of *ortho* congeners also have been demonstrated. In our studies, Aroclors and individual PCB congeners were evaluated for their effect on splenocyte viability and lipopolysaccharide (LPS)-induced splenocyte proliferation. The results suggested that mixtures of PCBs and/or noncoplanar congeners might be more immunotoxic than individual planar or mono-*ortho*-coplanar congeners. Since di- or multi-*ortho*-substituted congeners are found in high concentrations in human breast milk, we extended these initial studies to specifically investigate the relationship of immunotoxicity and chlorine substitution pattern. Results of these studies suggest individual congeners with chlorines in two or more *ortho* positions, regardless of surrounding chlorine substitutions, preferentially inhibit LPS-induced splenocyte proliferation as compared with non- or mono-*ortho*-substituted congeners. In addition, a widespread process in anaerobic sediments contaminated with PCBs, reductive dechlorination, could directly impact PCB substitution patterns by removing chlorines primarily from the *meta* and *para* positions, resulting in a modulation of toxic response. We have evaluated the toxicity of anaerobic PCB dechlorinated cultures using microbial communities from three PCB-contaminated soils. Our results indicate that preferential meta- and para-dechlorination was evident and that preferential ortho-substituted PCB congeners remained. In toxicity assays, these dechlorinated PCB mixtures were as or more toxic than the parent compound suggesting that ortho-substituted PCBs which preferentially bioaccumulate in the environment significantly contribute PCB toxicity. Our studies suggest a novel mechanism for the preferential inhibition of LPS-induced splenocyte proliferation by *ortho*-substituted congeners via an interruption of cell cycle progression through the decreased expression of the cell cycle regulatory protein, cyclin D2, which acts at a G_0/G_1 restriction checkpoint that allows progression into S phase. In addition, anaerobic reductive dechlorination, a naturally

occurring biological process that produces a PCB mixture with a high concentration of *ortho*-substituted congeners, also blocked cells at the $G_0/G_1/S$ interface by the inhibition of cyclin D2 expression. These results address the need to consider non-coplanar congeners within the context of risk assessment and further support the notion that "the use of Ah-receptor binding and its associated biological effects to assess the total toxicity of PCBs may no longer be defensible because of the actions produced by the non-coplanar congeners." Continued analysis of the molecular mechanisms of action of non-coplanar PCB congeners and their contribution to risk assessment connected with low-level exposures is warranted. An understanding of how the toxicity of specific PCB mixtures change after bioremediation and the mechanism behind this change in the laboratory will help determine the potential toxicity associated with PCB contamination in the environment.

Relevance

An inherent problem in environmental remediation is whether clean-up activities might alter, or even enhance, the toxicity of environmental chemicals for exposed humans. This study will address this important issue and investigate relevant mechanisms of immunotoxicity using a common contaminant (PCB mixtures) and a sensitive biological response, the immune system.

Objectives

We will examine potential immunotoxicity using two well defined assays: (1) *in vitro* proliferation of primary splenocyte cultures using either, the non-specific mitogen, LPS, or using anti-Ig cross linking of the B cell surface antigen receptor; and (2), *in vitro* differentiation of either the pro-B cell line, 70Z/3 with LPS which induces surface Ig expression, or LPS stimulation of the mature B cell line, WEHI, which induces B cell class switching and Ig secretion. In this manner, induction of proliferation in primary cell culture will be evaluated through either the B cell surface antigen receptor, Ig, or non-specifically through the use of the B cell mitogen, LPS. In addition, the use of *in vitro* derived cell lines will allow the analysis of the effect of PCBs on B cell differentiation.

Specific Aims 1. To analyze the effects of various PCB mixtures and individual congeners on NF-kB regulation and expression including IκB-α degradation.

Specific Aim 2. To analyze the effects of various PCB mixtures and individual congeners on apoptotic pathways including the Fas:FasL pathway, the BCL2 mitochondrial pathway, and the induction of early and late phase caspase enzyme activity

Specific Aim 3. To analyze the effects of various PCB mixtures and individual congeners on cell cycle dependent proteins including the retinoblastoma gene product (pRb), the cyclin dependent kinases (cdks), and their regulators.

Quarterly Accomplishments

Experimental Progress:

1. We have been working on optimizing our culture conditions for large-scale PCB cultures. One of the difficulties has been to reproduce the inhibition of LPS-induced proliferation observed in 96 well plates in large T25 flasks. This could be due to the enhanced surface area in T25 flasks versus a 96 well plate and the potential for PCBs to partition into the plastic.

2. We observed two key findings. First, in all cases T25 flasks appeared to "partition" the PCBs better than 96 well plates. Therefore, we began to optimize cell culture conditions utilizing large 6 well plates. This would allow us to generate bulk cultures with out resorting to the use of T25s. Second, to guarantee that these bulk cultures adequately represented conditions used in 96 well plates, we "removed" samples from these 6 well plates, transferred them to 96 well plates, and performed our usual 72-hour incubation. We found that the initial transfer of cells from the 6 well plates to the 96 well plates required the use of a glass syringe. Use of a typical micropipette tip was inadequate.

3. Our results indicate that the use of 6 well plates is representative of 96 well plates.

4. With this in mind, we have collected samples at 6 hr at 24hrs to investigate cyclin gene expression in cells treated with medium alone, LPS, LPS + 25 ppm Aroclor 1242 and 1 ppm Aroclor 1242. We have made both nuclear and cytoplasmic extracts from these cultures.

Performance Schedule and Status of Aims

In view of recent research results, our work in the last quarter has focused on the further investigation of the role of LPS in the mechanism of immunotoxicity associated with PCB exposure, specifically the signal transduction pathways associated with cell proliferation. Progress in accomplishing specific aims is on schedule.

3.2 Environmental Epidemiology and Risk Assessment Projects

3.2.1 Low Dose Radiation: Toxicological Models of Cancer Risk

Project Director: David G. Hoel, Ph.D.

Executive Summary

The use of experimental animals in radiation risk estimation is especially important for those situations when human data are inadequate or unavailable. This is particularly true for neutron exposures and low-dose rate exposures to gamma and x-ray. The purpose of this project is to apply biological based models to radiation risk estimation using experimental data.

Basic biological/mathematical models of radiation induced double strand chromosome breaks and misrepair have been developed and applied to the estimation of radiation risk of chronic myelogenous leukemia (CML), which is understood to be the result of a single specific translocation. Using this biomathematical modeling, it has been shown that CML risk estimates are considerably less that what is obtained from extrapolating to low doses some highly variable epidemiological data. Using the idea of susceptible stem cells it is also shown that the dose response is nonlinear at low doses. In addition, computer algorithms have been developed for biological based two stage mutation cancer models (Moolgavkar) for the analysis of lifetime mouse studies.

Relevance

By comparing the Moolgavkar risk models with the *in vivo* experimental data from the Argonne National Laboratory, the investigators will not only increase understanding of cancer development following low-dose radiation exposure, but also add biological credibility. This approach will provide a method for answering the important environmental question of whether risks are decreased with decreasing dose-rate, a key issue for chronic radiation control of workplace exposures.

Objective

The objective of this project is to determine the effects of dose-rate and radiation type on the development of various cancer types following low-dose radiation exposures. Two-stage biologically based Moolgavkar risk models will be used for analysis. Using previously validated data, assumptions made about the biological effects of ionizing radiation can be used in the two-stage model to predict dose-rate effects on the development of various cancers following low-dose exposures.

Specific Aim 1. To use the large Argonne National Laboratory Janus mouse study to answer basic questions concerning dose-rate and radiation type effects on cancer. This involves over forty thousand mice exposed acutely and chronically at several doses and using either gamma or neutron.

Quarterly Accomplishment

Project 1- The data analysis of the acute gamma exposed Sprague-Dawley rat studies has been completed and a manuscript is in preparation. The mouse data from Argonne labs was analyzed for gamma and neutron effects all seven major cites. This involved both acute and fractionated exposures. Dr. Hoel and Dr. Priest have completed the analysis of the beta and alpha exposed mouse studies from Harwell. Lung cancer was the endpoint and the RBE between alpha and beta turned out to be much smaller than what is believed by the radiation regulators. A manuscript has been prepared.

Performance Schedule and Status of Aims

Neither the performance schedule nor the status of aims has changed.

3.2.2 Low Dose Radiation: Epidemiological Risk Models

Project Director: David G. Hoel, Ph.D.

Executive Summary

The data used for estimating health risk from low LET radiation (e.g. x-ray, gamma) has been obtained from the A-bomb survivor cohort. This group, along with some cohorts of high dose medically exposed individual's makes up our source of information. Two important issues are of current concern: 1) Does the risk of cancer follow a linear dose-response at low-doses?, 2) Are individuals exposed at older ages (i.e. greater than 45 years) more susceptible to developing cancer than expected?

We have shown that the cancer risks at low-doses based upon the A-bomb data over estimates cancer risk. We have incorporated errors in dosimetry into the analysis of cancer risk and are proceeding to evaluate the risk at low doses of radiation exposure.

Relevance

Using Japanese A-bomb survivor data, the investigators seek to refine our understanding of the mathematical relationship between health outcomes (cancer) data and exposures to low-dose radiation. The issue of whether the relationship is linear or non-linear continues to be controversial. This project will address this very important scientific issue.

Objective

The shape of the dose-response function for radiation-induced carcinogenesis in humans has depended primarily on data obtained from the Japanese A-bomb survivors. This project will re-examine these data with respect to the linearity of cancer risks from low dose (1-10 rem) radiation exposures. An analysis of A-bomb survivor data for solid tumors and leukemia indicates that there is a non-linear relationship to carcinogenesis following low-dose radiation exposure. Uncertainty in the dose estimates, including underestimation of neutrons and a relative biological effectiveness (RBE) that varies with dose are being incorporated into this low-dose analysis. This comprehensive and focused analysis of epidemiological data from Japanese A-bomb survivors will greatly increased our understanding of the true epidemiological relationship between cancer risks and low-dose radiation exposure. In addition, DOE worker data which has been reported as providing the scientific basis for an increased susceptibility from exposure at older ages will be evaluated and contrasted with the A-bomb data.

Specific Aim 1. To carefully perform statistical modeling of the available epidemiological data from the A-bomb survivor cohort and the DOE worker cohort in order to increase our understanding of the cancer risk related to low-dose radiation exposure and the effect of older age on the magnitude of this risk.

Specific Aim 2. Epidemiological data from the A-bomb survivor cohort is being used to develop the biomathematical model of cancer risk. The previously published models for dose uncertainty and neutron exposure are being incorporated into our analysis. The DOE worker data from CEDER (DOE's data repository) will be used to evaluate the effect of older age cancer risk following low-dose radiation exposure. The entire set of available worker data will be modeled in order to evaluate the older age issue. The results of the worker analysis will then be compared to the analysis of the acutely exposed A-bomb survivors.

Quarterly Accomplishment

The epidemiological data involving bone sarcomas and head carcinomas from radium exposure was analyzed. The results showed that the best dose-response was a simple linear function. A manuscript has been accepted for publication.

Publication

Hoel, DG, Carnes B. Cancer Dose-Response Analysis of the Radium Dial Workers. Proceedings from the HEIR 2004 9th International Conference on Health Effects of Incorporated Radionuclides - Emphasis on Radium, Thorium, Uranium and their Daughter Products. GSF-National Research Center, Neuherberg, Germany, November 29-December 1, 2004. In press, 2005.

Performance Schedule and Status of Aims

Neither the performance schedule nor the status of aims has changed.

3.2.3 Health Risks of Low Dose Plutonium Exposure

Project Director: David G. Hoel, Ph.D.

Executive Summary

Human data on health risks associated with internal exposure to radionucleides (by inhalation and/or ingestion) is limited. With regard to plutonium exposures, there have been two DOE worker studies and, more recently, several rudimentary studies of Russian nuclear workers. One of the DOE worker cohorts (Los Alamos) contains data that may be very useful in understanding the carcinogenic effects of low-dose plutonium exposure. In contrast to the paucity of human data, there is a considerable amount of experimental data related to the development of cancer in rats and dogs following plutonium inhalation. A statistical model of cancer risk following low-dose plutonium exposure is becoming increasingly important with respect to planned DOE material disposition activities, both domestic and international. For example, plans to eliminate surplus U.S. plutonium during the next two decades, through the irradiation of mixed oxide fuel and the conversion of a certain portion of the material to an immobilized waste form, represent significant program initiatives, the effects of which should be incorporated into evolving statistical risk models. U.S. data will be related to prior studies of the Mayak workers which have consistently shown a higher level of lung, liver and bone cancer in comparison to U.S. workers. Pulmonary fibrosis is also a risk from the inhalation of plutonium; factors related to this risk will be assessed through the analysis of available animal and human data.

Relevance

The processing and storage of plutonium requires a quantitive understanding of the health risks of plutonium, particularly in the low-dose range. Furthermore, DOE workers who may be exposed to plutonium should be monitored with a state-of-the-art medical surveillance program that includes the use of validated biomarkers.

Objectives

1. The general problem we are considering is the evaluation and protection of the health of DOE workers in their handling of plutonium at the SRS and other DOE facilities. The project will begin by developing risk models of the health effects of low dose exposures and the design of an appropriate medical surveillance system.

2. The first step will be a quantitative evaluation of the human and animal data so that we have good productive risk models.

3. Secondly, we will develop a medical and environmental surveillance system which includes the use of film badges for measuring external radiation dose and urine analyses for the measurement of internal plutonium levels.

Specific Aim 1. To develop a medical surveillance system for DOE workers. This includes methods for the medical and environmental surveillance of the workers as well as up to date quantitative health risk models of plutonium exposure.

Quarterly Accomplishment

The animal data for plutonium continues to be collected and prepared for analysis.

Performance Schedule and Status of Aims

Neither the performance schedule nor the status of aims has changed.

3.2.4 Population Risk Studies Using Geographic Information System Technology

Project Director: Daniel Lackland, Dr.P.H.

Executive Summary

We have developed the infrastructure, resources and technical expertise necessary to conduct epidemiological assessments of population health risks using a geographical information system (GIS). Our sources include the following:

Savannah River Region Health Information System (SRRHIS)
The geographic cancer registry incorporates 25 counties around the Savannah River Site. Cancer incidence data obtained in a high quality manner is an essential component of epidemiological investigations.

A direct link to this resource has been established in which cancer cases are geographically identified and incorporated in the data analysis. SRRHIS provides the cancer-related component of the assessment system. Cancer incidence and mortality rates are analyzed with respect to various aspects of population characteristics, demographic data and environmental exposures.

Geo-coding System
The ability to ascertain and analyze health-related, environmental, and socio-economic data for small areas, such as a census block, is an essential component of epidemiological investigation. A Geographic Information System (GIS) defines geographic study areas by

organizing small areas such as census blocks. The system consists of computerized databases structured to a defined geographic area combining the tools for thematic map generation, proximity analysis, buffer zone identification and map overly comparisons.

A critical component of any GIS is the ability to "address match" other databases into the system. An efficient GIS with a high match record must incorporate a system to add new addresses and changes, which requires an elaborate system of updates. In addition to collecting new data, epidemiological investigations are greatly enhanced with the use of existing data, saving money and time. Such databases, however, must be comprehensive and include multiple health outcomes, co-morbidities, indicators of socio-economic status, environmental exposures and population demographics and characteristics.

The analytical assessment of disease patterns constitutes a critical stage in the investigation of the environmental etiology of disease. The assessment involves the use of resources such as the GIS and multiple databases. Analyses involve a complex and sophisticated quantitative methodology.

Existing Databases
The Project has established access links to various health and environmental data bases including the SC Medicaid and Medicare data bases, hospital discharge and billing data, census TIGER files, as well as data and tissue specimens from cohort studies such as the Evans County Heart Study. The Project also maintains the capability to collect new data and tissue samples.

Objectives

1. To develop a comprehensive population risk assessment system and associated protocols.

2. To conduct several epidemiology risk assessments of populations in the vicinity of the Savannah River Site (SRS) using the resources of the comprehensive system.
3. To establish and maintain a state-of-the-art information system that interfaces with the agencies and custodians of health, environmental, geographic demographic and economic databases in order to provide more accurate and comprehensive population risk assessment.

Specific Aims

Specific Aim 1. To continue to develop and enhance the Geographic Information System as a tool for the conduct of population risk studies.

Specific Aim 2. To continue the analysis of population cancer risks in the vicinity of the Savannah River Site (SRS).

Specific Aim 3. To assess population health risks in relation to specific environmental hazards at SRS and other DOE sites.

Specific Aim 4. To assess health risks of former workers at the Savannah River Site.

Quarterly Accomplishments

1. The Toxic Release Inventory from DHEC has been installed, and these data as related to TCE sources are being plotted with particular emphasis to the Savannah River Region.

2. The cancer rates in the Savannah River Region have been updated for 1997-2001 with significant changes in cancer incidence identified. These incidence changes are undergoing further analysis with respect to standardized mortality rates and geographical distribution.

3. Poverty levels have been mapped in conjunction with the geographic patterns of cervical cancers.

4. GIS instruction continues be a part of the Design and Conduct of Epidemiologic Studies graduate student course with project investigators as the lead instructors.

5. Planned manuscripts and abstracts include: 1] an assessment of population disease rates and the disease rates of former workers; 2] an assessment of environmental exposures and Parkinson's Disease; 3] the geographic comparisons of adverse outcomes and the availability of primary medical care; 4] cancer rates and the location of cancer prevention services 5] cardiovascular disease among retires SRS workers 6] cancer rates for former SRS workers

Performance Schedule and Status of Aims

Neither the performance schedule nor the status of aims has changed.