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RESEARCH TECHNIQUES AND METHODS FOR THE DETECTION AND PREVENTION OF CARCINOGENESIS IN THE INDUSTRIAL WORKER

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UNIVERSITY OF LOUISVILLE
HEALTH SCIENCES CENTER
SCHOOL OF MEDICINE
GRADUATE SCHOOL
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REPORT FOR THE
CHEMICAL MANUFACTURERS ASSOCIATION
(FORMERLY MANUFACTURING CHEMISTS ASSOCIATION)

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RESEARCH TECHNIQUES AND METHODS

FOR THE DETECTION AND PREVENTION OF

CARCINOGENESIS IN THE INDUSTRIAL WORKER

Program Final Report

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INTRODUCTION

In 1974 Dr. John L. Creech, Jr., a surgeon and the plant physician for B.F. Goodrich's Chemical Plant on Bells Lane in Louisville, Kentucky, identified the increased occurrence of hepatic angiosarcoma in workers involved in polyvinyl chloride manufacturing. This discovery led to the implementation of an industrial cancer control, detection and prevention program at the Louisville B.F. Goodrich Plant involving approximately 1,800 workers: 1,200 were active employees and 600 were previously employed individuals. The University of Louisville, in cooperation with the B.F. Goodrich Company, developed and implemented the program and has continued to follow this cohort of workers clinically and epidemiologically.

A proposal submitted to the Chemical Manufacturers Association, formerly the Manufacturing Chemists Association, to foster scientific research in better technical methods for the detection and prevention of chemical injury and carcinogenesis in industrial workers was awarded in 1976. These investigations covered nine general areas of interest and involved both animal and human studies. The studies included: (A) the use of the human immunological system for the detection of injury from vinyl chloride and other chemicals, (B) the assessment of hepatic biochemical enzymatic systems to detect and characterize vinyl chloride and other chemical injury, (C) the use of tissue and urinary glycosaminoglycan changes for early detection and diagnosis of chemical injury including cancer development, (D) the histological evaluation of liver tissue from chemical workers and the assessment of its collagen content as an indicator of chronic latent injury, (E) synthesis and analysis of vinyl chloride intermediate and end products to further understand its biological metabolism and removal, (F) the identification and assessment of cell assays to determine the carcinogenic potential of industrial chemicals, (G) the evaluation of tissue antigenic systems for detecting chemically-induced carcinogenesis, (H) exploratory studies for the use of isolated human liver cells to determine individual ability to detoxify chemicals, and (I) the further assessment of vinyl chloride metabolism by the use of whole body autoradiographic technique.

Each of these areas of study were directed toward four major objectives: (1) to better characterize and understand the pathogenesis of chemically-induced injury, especially vinyl chloride injury, (2) to characterize the mechanisms of chemical injury and the body's biological defense to this injury using animal studies, (3) to evaluate and assess old and new biochemical parameters for the detection and verification of chemical injury in humans, and (4) to develop new techniques that could be applied to the problem of identifying occupationally-related chemical injury for purposes of prevention.

Many of the original objectives of the individual studies have been changed or modified because of developments and discoveries in both the animal

program and the human medical surveillance program which were ongoing concomitantly during the research periods of this study. This has led to a large body of literature which has changed the medical approach to the screening, identification and verification of occupationally-related chemical injury.

Although the contractual funding of this grant was only three years, each year was funded separately with varying time intervals between the funding from year to year so that almost six years have evolved between the initiation of these studies and this final report. This report, therefore, summarizes the work accomplished during this six-year interval and the related work which is still ongoing.

The investigators' reports vary in the degree of detail depending upon wnether their work is in the process of being published or has already been published. Work not yet published is described in greater detail; published work is accompanied by a copy of the actual article(s) attached in the Appendix.

The investigators wish to express their appreciation to the B.F. Goodrich Company and especially to the entire management of its Bells Lane, Louisville plant. The continued cooperation and help of the plant managers, Philip Lawrence, Gabriel Le Febvre, Edward L. Beeler and especially Raymond Pruitt, Personnel Manager, along with Dr. John Creech, Jr., and the medical department staff, requires special acknowledgement.

The investigators also wish to thank their laboratory and research staff for their devoted work, and to thank Dr. Harold Boyer, Vice President for Health Affairs, and Dr. Joseph X. Musacchia, Dean of the Graduate School, for their administrative efforts, and especially, Mrs. Vicky Strong for her patient secretarial support.

A special thanks is given to Dr. Kathleen O'Connell without whose help and support completion of the periodic and final reports would have been much more difficult.

Finally, and very importantly, the participation and cooperation of the entire work force of the B.F. Goodrich Bells Lane Plant, and their representative unions--the Distillery Workers Union, the International Brotherhood of Electrical Workers #369, the International Association of Machinist Workers #681, and Pipefitters Union Local #522--is acknowledged with appreciation. This work would have been impossible without the active involvement and help of the workers. The objectives of this task were all directed toward improving and maintaining the best possible work environment.

Again, a thank you to all who have contributed to this effort.

SUMMARIES OF
RESEARCH PROGRAMS

PROGRAM A

STUDIES OF HUMAN IMMUNOLOGICAL SYSTEMS IN THE DETECTION OF VINYL CHLORIDE AND OTHER CHEMICAL INJURY; Investigators - H. P. Fortwengler, Jr., and C. H. Tamburro

A1. Evaluation of Immunocompetence of Humans Chronically Exposed to Vinyl Chloride

The scientific literature is replete with the demonstrations of immunodepression as a manifestation of medical disease, especially cancer. Lymphocytes (T cells) can be cytotoxic to human tumor cells and are often found decreased or poorly functioning in cancer patients resulting in various degrees of immunodepression. The immunocompetence of a cohort of chemical workers was studied to determine if there was any evidence that (a) varying degrees of prolonged exposure to vinyl monomers was associated with immunological suppression, (b) the immune response was impaired in workers with exposure-related liver injury including angiosarcoma, and (c) exposure caused changes in immunological function which were identifiable in the pre-cancerous stages. The immunocompetence of 75 employees with demonstrated liver disease and 225 individuals without clinical or laboratory evidence of disease was studied. No significant clinical or laboratory evidence of impaired immunocompetence was found in the immunological parameters studied, nor were differences seen among the various subpopulations of workers.

A2. Assessment of the Leukocyte Adherence Inhibition Test for the Detection of Angiosarcoma of the Liver

Halliday et al. (1974) and Thompson (1976) had reported the development of a leukocyte adherence inhibition test which had identified the clinical stages in colon cancer. Similar findings were reported in individuals with hepatic cancer. This study was undertaken to determine the feasibility of applying this newly developed technique to screen vinyl chloride-exposed workers for angiosarcoma of the liver. It was assessed in a number of chemical and nonchemical workers who had cancer. This test system lacked sufficient reproducibility to be considered for clinical use.

A3. Assessment of Vinyl Chloride-Induced Angiosarcoma Antigen for the Detection of Latent Angiosarcoma in Exposed Workers

New antigens arise from tumors formed as a response to carcinogens. Their presence in methylcholanthrene-induced sarcomas was discovered by Foley in 1953. This discovery in mice was verified and extended by Prehn and Main (1957) to conclude that there were antigens particular to and specific for tumor tissue. Subsequent evidence of tumor antigen was found in humans by the Hellstroms, Vankey, Halliday, Maluish, Thompson, and others. The majority of

evidence suggested that the tumor antigens found were distinctive for each histological type of tumor. Studies were undertaken to determine whether angiosarcoma tumor antigens would provide specific immune reactions that could be utilized as a specific test for vinyl chloride-induced tumor development. Lymphocytes from normal and liver diseased individuals were isolated and then grown in the presence of a liver reagent prepared from either normal individuals or individuals who had angiosarcoma. Reactivity of lymphocytes was assessed by determining the incorporation of ^{3}H-thymidine into stimulated cultures as compared to unstimulated cultures. A comparison study was performed between vinyl chloride plant workers and nonchemical plant workers utilizing reactions of lymphocytes to normal and angiosarcoma liver reagents. The lymphocytic response was also studied in vinyl chloride-exposed and non-vinyl chloride-exposed workers with and without evidence of liver disease. Initially, specific reactivity was seen only among vinyl chloride workers. Although these inital results were provocative, subsequent studies with large control populations demonstrated similar reactivities. The lack of further occurrences of angiosarcoma in the cohort worker population prevented further characterization of the tumor antigen and restudy of the earlier findings.

A4. Study of Histocompatibility Leukocyte Antigen (HLA) Frequencies in Chemically-Exposed Workers

This study involved the search for HLA tissue types which may identify individuals susceptible to chemically-induced injury. In 1976 Mulvihill suggested genotyping as a means of screening potential employees for antigen subtypes which may predispose them to neoplastic development after occupational exposure. It was prompted by the suggested increased occurrence of HLA-B27 antigen in workers with occupational asbestosis. The HLA frequencies in polyvinyl chloride manufacturing workers were compiled to determine if any increased genotype occurrences were associated with angiosarcoma or other chemically-related liver disease. Nine hundred individuals were eligible for this study: 538 individuals were HLA tissue typed and 30 different HLA-A, HLA-B, and HLA-C antigens were assessed. Preliminary comparisons were made with the HLA frequencies in vinyl chloride workers with and without liver disease, and later in those with liver disease of chemical vs. nonchemical origin. HLA frequencies were also analyzed in those individuals with and without biochemical evidence of liver injury. HLA-B15 was found to occur with a greater frequency among chemical workers with liver disease than in "control" and "normal" populations. The HLA-B15 occurrence was greater among those with chemical liver injury, although it was not statistically significant. Two other HLA markers occurred with unusual lower frequency.

A5. Identification of the Endothelial Cell as the Cell of Origin For Vinyl Chloride Angiosarcoma of the Liver

There has been considerable disagreement as to the cell of origin for liver angiosarcoma, a nonparenchymal cell malignancy of the liver. This is of

great importance since the identification of the type of cell responsible for malignant transformation could provide the basis for a better understanding of chemically-induced carcinogenesis. This was particularly relevant to vinyl chloride-induced liver cancer since vinyl chloride is predominantly metabolized and detoxified by hepatocytes and produces a malignancy of the liver that is nonparenchymal in origin. Considerable controversy arose as to whether the cell of origin was a macrophage (Kupffer cell), a fibroblast, or an endothelial vascular lining cell. The recent identification of Factor VIII production by endothelial lining cells led us to determine whether liver angiosarcoma tumor contained increased amounts of coagulation Factor VIII. Factor VIII is a large protein, often called antihemophilic factor, found differentially in endothelial cells, platelets, and megakaryocytes. The studies described herein demonstrated Factor VIII presence in endothelial lining cells of arterial and venous vessels in normal liver, as well as in the cells of angiosarcoma. Histological verfication of the cell type was determined by light microscopy of the same tissue sections of the tumor. This verified the endothelial cell as the cell of origin in angiosarcoma.

PROGRAM B

STUDY OF HEPATIC BIOCHEMICAL AND ENZYMATIC SYSTEMS FOR THE IDENTIFICATION OF VINYL CHLORIDE CHEMICAL INJURY AND CANCER DEVELOPMENT: ANIMAL AND HUMAN STUDIES; Investigators - J. T. Du, M. T. Tseng, and C. H. Tamburro

B1. Characterization of Hepatic Enzyme Changes in Rats With Prolonged Vinyl Chloride Exposure: Decreased Glucose-6-Phosphatase Activity

Considerable concern was initially raised concerning the best technique for screening individuals with chemical exposure. At present, federal testing requirements recommend enzyme studies related to liver parenchymal cells. However, clinical evidence indicated that not only were the hepatocytes injured but so were Kupffer and endothelial cells, and that the endothelial cell or Kupffer cell was the malignant cell of origin. In addition, studies to determine the sequential enzymatic changes which occurred with prolonged continuous vinyl chloride exposure similar to that seen with the worker population were needed. Most studies at this time had been conducted with short term exposures. The early animal studies included assessment of the standard clinical, biochemical, and enzymatic studies and later explored the enzymatic changes occurring in various subcellular organelles. These included (a) markers for microsomal enzymes related to the metabolism of vinyl chloride, P-450, NADPH-cytochrome reductase, and mixed function oxidase, (b) cytosolic enzymes related to glutathione metabolism and glutathione content, (c) mitochondrial marker cytochrome-C-oxidase, (d) cytosolic enzymes glucose-6-phosphatase, glucose-6-phosphate dehydrogenase related to pentose phosphate pathway, and (e) nucleic acid synthesis. In addition, protein synthesis was evaluated by *in-vitro* incorporation of ^{3}H-leucine. These

studies were conducted in sequential experiments exposing Sprague-Dawley rats to 10,000 ppm vinyl chloride for a period of up to 300 exposure hours. These studies demonstrate various adaptive changes in liver parenchymal cells that were not identifiable by conventional clinical laboratory test (CCLT). These data illustrate the limited usefulness of CCLT in identifying early liver cell changes even during high levels of exposure.

B2. Morphological Alterations in Livers of Rats Exposed to Vinyl Chloride

Sequential morphological assessments were performed in the rats from both chronic exposure experiments utilizing light and electron microscopy. Light microscopic findings included an increase in liver cell polyploidy, double nucleated cells, and areas of focal hepatocellular hyperplasia without evidence of cellular injury or increased fibrosis. Electron microscopic changes included proliferation of smooth endoplasmic reticulum without evidence of other subcellular organelles, or increased collagen deposit in the Space of Disse.

B3. Alterations of Oxidizing and Detoxifying Systems of Rat Liver After Prolonged Exposure to Vinyl Chloride

A second long-term study, based partly on the results from the B1 study, was conducted to determine the adaptive capability of the detoxifying systems of liver cells. Glutathione reductase activity, glutathione content, glutathione epoxide-S-transferase (GEST; Glutathione transferase E), and glutathione aralkyl-S-transferase (GAST; glutathione transferases A&B) were measured in rats exposed to vinyl chloride and to chloroethanol, a vinyl chloride metabolite. A third set of studies was conducted to compare these detoxifying enzymes in rats exposed to vinyl chloride and excessive alcohol consumption. These studies illustrated the adaptability of liver cells to prolonged exposure to xenobiotics. Liver cells showed increases in reduced glutathione and glutathione reductase activity and subsequent increases in GEST and GAST, reflecting a progressive increase in the liver cells' capacity for detoxification of vinyl chloride's toxic intermediates. Concomitant alcohol consumption appears to interfere with this detoxifying capability and appears to place the hepatocyte at greater risk of malignant transformation.

B4. Oxidative and Detoxifying Ability of the Liver Mesenchymal vs. Parenchymal Cells in the Metabolism of Xenobiotics

Another aspect of the prolonged vinyl chloride exposure studies was to determine the oxidative and detoxifying capability of various liver cells. In collaboration with Dr. Feldhoff, the oxidative and detoxifying ability of the liver mesenchymal versus parenchymal cells was studied by determining P450, glutathione reductase, GEST, and GAST in liver hepatocytes and endothelial cells.

The mesenchymal cells were shown to be capable of oxidizing xenobiotics which require P-450 and mixed function oxidases, although this capability was less than that of the parenchymal cells by a 70 to 1 ratio. GEST and GAST activity were also present but in the ratio of 1 to 65, and 1 to 500, respectively. These results demonstrate that the nonhepatocytic liver cells have the capability to activate vinyl chloride and other similar xenobiotics, although to a lesser degree than hepatocytes. However, their ability to detoxify vinyl chloride metabolites is significantly less and may contribute to the reason for nonmesenchymal cell malignant transformation.

B5. Effectiveness of Indocyanine Green (ICG) Clearances in the Detection of Liver Injury

A chemically-exposed worker cohort of almost 1,000 individuals were medically screened to determine the effectiveness of ICG clearances versus standard biochemical studies of the liver to identify latent hepatic injury. Alanine aminotransferase (ALT/SGPT), aspartic aminotransferase (AST/SGOT), gamma glutamyl transpeptidase (GGPT), alkaline phosphatase (AP), and total bilirubin (TB) were assessed in contrast to 3 different dose levels of ICG. Positive predictive values as well as the specificity and sensitivity of each of the screening tests were determined in "normal" and "abnormal" worker populations as well as in those with hepatic disease and nonhepatic disease. Chemical and nonchemical liver injury were also determined in analysis of their relationship of work histories to the biochemical abnormalities. These studies demonstrated that ALT/SGPT was the screening test with the highest sensitivity and specificity among federally-required studies. ICG clearance, even at the low dose, clearly remained the best overall screening test for the detection of subclinical hepatic disease.

B6. Assessment of Bile Acids vs. Indocyanine Green (ICG) Clearances in The Detection of Liver Injury Due to Chemical Exposure in The Human Population

Fasting serum bile acid levels and ICG clearances were performed for 400 employees with varying degrees of chemical exposure as well as biochemical injury related to vinyl chloride exposure. Bile acid levels, ICG clearances and standard enzymatic biochemical tests were assessed regarding their ability to identify normal individuals, individuals with liver disease of nonchemical origin, and individuals with liver disease with chemical origin, correctly. These more sensitive studies were to identify early chemical injury; six other enzyme studies were also conducted regarding more specific enzymes for hepatocellular injury, such as sorbitol dehydrogenase. One thousand seven hundred and sixty human seras taken from 900 exposed workers were assessed and the results compared to the vinyl chloride exposure.

Fasting serum bile acids demonstrated a high sensitivity separating normal versus abnormal individuals with early or latent liver dysfunction, particularly chemically-induced types of injury. Fasting serum bile acids

hold promise as a potential detector of early liver injury, whether chemical or not. Bile acids have the advantage of being natural biological substances which may be taken orally for screening clearance studies.

PROGRAM C

STUDY OF GLYCOSAMINOGLYCAN CHANGES IN THE EARLY DETECTION OF HEPATIC FIBROTIC INJURY IN CHEMICAL EXPOSURE AND HEPATIC CANCER DEVELOPMENT; Investigators - C. E. Kupchella and R. Warick

ANIMAL STUDIES:

C1. The Study of Glycosaminoglycan Changes in Livers of Animals Bearing Metastasizing Hepatomas

C2. Significance of Tissue Glycosaminoglycan Elevations in Hepatic Necrosis and Regeneration

Animal studies to determine the sequence of GAG changes in experimentally-induced fibrotic liver injury, and the relationship between GAG patterns in tumor tissue and in urine were studied in animals with fast vs. slow growing, metastasizing vs. nonmetastasizing, chemically-induced and transplantable liver cells tumors.

The experimental animal studies identified heparan sulfate (a type of GAG) elevation in hepatic tissue undergoing fibrotic changes and that the increased levels were reflected in the urine. Both heparan sulfate and hyaluronic acid levels were 3-4 times higher in experimentally transplanted liver tumors than in normal controls. Urinary excretion reflects both tumor GAG composition and size. Livers from animals bearing metastasizing hepatomas had a 10-fold great concentration of nonsulfated neutral uronic acid positive material than animals bearing nonmetastasizing hepatomas. Finally, hepatic necrosis was shown to be accompanied by significant tissue GAG elevation; hepatic regeneration was not.

HUMAN STUDIES:

C3. Characterization of Glycosaminoglycan Patterns in Humans with Angiosarcoma

C4. Evaluation of Glycosaminoglycan Analysis of Urines of Chemically-Exposed Workers

C5. Characterization of Glycosaminoglycan Patterns in the Identification of Chemical and Nonchemical Injury of the Liver

The study of glycosaminoglycans, glycoproteins involved in wound healing and scar formation, as potential indicators of chemical injury, fibrosis, and cancer development was conducted in a large cohort of exposed workers. Analysis of GAG in tissue and urine was performed in individuals with angiosarcoma and heptocellular tumors, in individuals with chemical and nonchemical liver injury, and in individuals with chemical liver abnormalities and in normal controls. The studies indicated that human hepatic angiosarcoma and fibrotic liver disease are accompanied by elevated tissue GAGs, that the tumor tissue is different from the adjacent fibrotic areas of the liver, and that patients with angiosarcoma and primary hepatocellular carcinoma (hepatoma) have characteristic urinary GAG patterns. These patterns are not found in "normal" individuals. Urinary GAG determinations give a better indication of liver disease than ultrasound or radioisotopic scanning, and although they are not as sensitive as transaminases or ICG clearances, fractionated GAG analysis appears to differentiate between active and inactive liver disease.

PROGRAM D

HISTOLOGIC AND MORPHOMETRIC ANALYSIS: A MEANS OF ASSESSING HEPATIC INJURY IN CHEMICAL WORKERS; Investigators - G. H. Barrows, G. R. Schrodt, and C. H. Tamburro

D1. Systematic Assessment of Histological Lesions Characteristic of Vinyl Chloride or Vinyl Monomer Injury

D2. Computer-Assisted Morphometric Analysis as a Means of Determining Collagen Content

D3. Development and Assessment of Morphometric Method of Analysis of Collagen Content from Liver Biopsies - Normal Occurrence with Age

Vinyl monomer injury has been associated with various hepatic histological abnormalities. These have included subcapsular, portal, and perisinusoidal fibrosis, as well as hyperplasia of the hepatocytes and nonparenchymal (sinusoidal) cells. The ability to assess light microscopic determinations of fibrosis (liver tissue collagen content) using computer-assisted morphometric analysis quantitatively, was delineated. The methodologies determined the normal presence of collagen and the effect of age. Prospective analysis to determine the ability of specific histological findings to correctly identify vinyl monomer exposure was also done.

Both manual and computer-assisted morphometric assessment of normal collagen content identified the distribution between perisinusoidal,

periportal, and pericentral areas in 4 different anatomical locations of the human liver of 4 different age groups. These studies demonstrated that the collagen content of the liver varies with age, increasing after the fourth decade. The collagen distribution shows an increased desposition in the perisinusoidal and midzonal regions with age. The computerized morphometric readout demonstrated that the collagen estimates vary from 2-6% of the total liver globules. (This does not include capsular fibrosis or collagen support tissue about major vessels.)

D4. Light Microscopic Assessment of Liver Tissue Obtained from Vinyl Chloride Workers Characterizing Various Histological Lesions

Light microscopic assessment of liver tissue obtained from vinyl chloride and nonvinyl chloride workers illustrated that focal hepatocellular hyperplasia and focal mixed hyperplasia, sinusoidal dilitation and focal areas of increased reticulum characterized chemical injury could be identified in a prospective blind study and that those individuals with these identified lesions had the highest correlation with their vinyl chloride accumulative exposure ranked months; this correlation was not seen with other chemicals within their exposure environment.

PROGRAM E

STUDIES OF VINYL MONOMER CHEMICALS AND THEIR METABOLITES USING CHEMICAL STRUCTURE AND SYNTHESIS IN THE DETERMINATION OF TOXICITY OF THESE AGENTS; Investigator - J. L. Wong

E1. Synthesis, Purification, and Utilization of Vinyl Chloride Metabolites in Mutagenicity and Carcinogenicity Studies

E2. Detoxification Studies of Vinyl Chloride and Its Metabolites

E3. Vinyl Chloride Metabolite Detection--Chloroacetic Acid

In order to further elucidate the intermediate metabolism of vinyl chloride, especially with regard to the carcinogenic potential of its intermediate metabolites--chlorooxirane and chloroacetaldehyde--laboratory synthesis of these two putative metabolites was performed. The materials were utilized to further study the detoxification of these agents and their effect on bacterial systems. Additional studies were conducted to determine the ability to identify chloroacetic acid by mass spectrometry and gas chromatography.

These studies illustrated that although chlorooxirane and chloroacetaldehyde eventually give the same final product, the rate of reaction and the

intermediates in the two reactions are different. Chlorooxirane conjugates instantaneously with the sulfhydral compounds, while chloroacetaldehyde takes about 2 1/2 hours for a comparative reaction. Although both routes are converged to yield the cysteine-S-acetaldehyde conjugate, the reaction rates are vastly different, one taking minutes, the other hours to complete. Chloroacetic acid is identifiable by mass spectrometry in liquid solutions, solutions as small as 0.02M (2 mg/ml), while gas chromatography sensitivity was limited to concentrations of 0.2 to 2 mg/ml H_2O (200 to 2,000 ppm). Use of a porous polymer solid support and formic acid is expected to improve sensitivity by 100-fold. Combination of GC-MS techniques appear to be the most promising approaches to the detection of end metabolic products and biological tissue.

PROGRAM F

ASSESSMENT OF ASSAYS FOR THE CARCINOGENIC POTENTIAL OF INDUSTRIAL CHEMICALS USING PROKARYOTIC AND EUKARYOTIC SYSTEMS; Investigators - U.N. Streips and G. Sonnenfeld

F1. Further Development of Bacterial Systems for Testing Carcinogenicity and Mutagenicity of Chemical Agents Used in the Manufacturing of Vinyl Chloride and Synthetic Rubber

Application of mutagenic assays for the determination of mutagenic potential of a list of environmental chemicals was conducted using improved modifications of their bacterial system. In addition, a new screening technique for carcinogenesis was developed involving the inhibition of interferon induction. This was applied to several carcinogens and mutagens and compared to the effectiveness of the standard viral bacteriological assays. These studies have demonstrated improved techniques for rapid screening of large numbers of chemical formulations being utilized within an environmental region.

F2. Preliminary Studies on the Use of Interferon Induction as an Indicator of Mutagenicity and Carcinogenicity of Chemicals

The new method assessing the mutagenic/carcinogenic capabilities of chemicals using an interferon assay is reported as well as related research regarding the controlled process of cell division in bacteria and how these findings relate to mammalian cells.

Interferon induction inhibition in contrast to the Ames bacterial assay demonstrated the ability to separate more accurately agents with more carcinogenic potential related to human occurrences.

PROGRAM G

THE STUDY OF LIVER TISSUE ANTIGENS AND ANTIBODIES IN THE DETECTION OF VINYL CHLORIDE INJURY; Investigator - E. Espinosa

G1. The Study of Tissue Antigens From Liver Tumors, Angiosarcoma, and Hepatomas

G2. Circulating Antigens and Autoantibodies in Vinyl Chloride-Associated Liver Disease

The approach to the use of tissue antigens produced by neoplasms and identified in serum, either as the antigens or autoantibodies, were studied in angiosarcoma and in chemically-induced liver cancers. Several normal tissue antigens, a tumor-associated protein antigen, and a glycoprotein liver antigen absent in individuals with angiosarcoma were identified and characterized. The finding of a missing antigen in vinyl chloride-related angiosarcoma stimulated studies of antigenic deletion in chemically-induced hepatoma and cultured human liver carcinoma cells. Two liver antigens were found to be absent. One of these antigens was shown to be normally present in other tissues (kidney and spleen) in addition to the liver. The second antigen was detected only in the liver and was found to be unrelated to the liver-specific F antigen. Liver-specific F antigen was also studied as a possible sensitive indicator of chemically-induced liver tumor. F antigen appeared to be absent in fast growing hepatoma 7777 and undetectable or very low in the slow growing hepatomas. The level of the F antigen did not appear to correlate with the rate of growth of these tumors; however, an increased concentration of F antigen appears related to those tumors with the highest metastatic characteristics. Studies of cultured human cancer carcinoma cells also noted a deficiency in liver-specific F antigen similar to that of the experimental hepatoma 7777. These data support the clinical observation that tissue antigens appear to be more useful in treatment and follow-up care, while antigenic deletions may prove more important for screening and early detection.

PROGRAM H

USE OF ISOLATED MAMMALIAN LIVER CELLS FOR THE STUDY OF CHEMICAL MONOMER METABOLISM; Investigator - R. C. Feldhoff

H1. Isolation of Mammalian Liver Cells for the Study of the Metabolism of Chemical Monomers

H2. The <u>In Vitro</u> Study of Albumin Synthesis by Liver from Human Biopsies

Presently, the rat has provided the most useful means of studying hepatic metabolism in mammalian cells. The intact liver, however, consists of at least four cell types which differ in their metabolic and functional characteristics. These studies were directed toward the development of clinically utilizable techniques to study human liver biopsy material in relationship to its ability to synthesize, retain, and secrete protein.

Improved techniques were also utilized for isolation of hepatic parenchymal and nonparenchymal cells so that nearly homogeneous populations of cells could be studied for their oxidizing and detoxifying capabilities. Preliminary results of this late addition to our research techniques have proven encouraging in the adaptability of highly sophisticated laboratory techniques to the study of human tissue in an in vitro system. It has demonstrated that animal and human tissue obtained by a clinical biopsy technique are viable in an in vitro system, being able to perform normal functions relative to albumin synthesis. The technique for the study of this tissue in an in vitro system may be applicable to human tissue from individuals with varying degrees and types of cellular injury. This technique would provide the ability to determine the oxidizing and detoxifying capability of liver cells to various xenobiotics in a quantitative fashion. Although very futuristic in its approach, it appears accomplishable since the present developed methodology is clinically applicable.

PROGRAM I

THE STUDY OF TISSUE DISPOSITION OF INDUSTRIAL CHEMICALS: THE VINYL CHLORIDE EXAMPLE; Investigators - W. J. Waddell and C. Marlowe

II. The Use of Whole Body Autoradiography in Specific Tissues. Localization of Accumulated and Retained Industrial Chemicals and Their Metabolites

The need for methods which more accurately identify the sites of localization of chemical agents and their metabolites has obvious importance. The use of whole-body autoradiography to identify the location of radioactively tagged chemicals was studied with regard to ^{14}C-vinyl chloride. Whole-body saggital sections of mice exposed for three hours in ^{14}C-vinyl chloride demonstrated that the highest levels of radioactivity in mice sacrificed at 20 minutes and one hour after removal from the vinyl chloride environment were observed in the liver, pancreas, kidney, intestinal contents, urine and bile. Concentrations of metabolites in the organs of excretion decreased continually over a 24-hour period. After nine hours removal from the vinyl chloride environment, Harder's gland, epithelium of the esophagus and intestine, and sublingual glands retained the highest levels of radioactivity, while moderate concentrations were seen in the liver, kidney, and intestinal contents. At 24 hours, the primary organs of retention were the thymus, Harder's gland, liver, and esophagus and intestinal epithelium. The high concentration of

nonvolatile metabolites of vinyl chloride retained in the thymus after 24 hours suggested that there was covalent binding of these metabolites to the molecules in the thymus. Possible interactions of the thymus may reflect a dual mechanism of carcinogenic action, one related to tissue damage in the liver, the other to a suppressed immune surveillance system.

RESEARCH PROGRAMS AND RESULTS

PROGRAM A

STUDIES OF HUMAN IMMUMOLOGICAL SYSTEMS IN THE DETECTION OF VINYL CHLORIDE AND OTHER CHEMICAL INJURIES; Investigators - H.P. Fortwengler, Jr., and C. H. Tamburro.

A1. Evaluation of Immunocompetence of Workers Chronically Exposed to Vinyl Chloride

Background

The study of the human immunological systems as a means of detecting chemical injury was initiated to determine whether or not the body's ability to recognize substances that were foreign would make specific responses that could be identified clinically. The immune response can be divided loosely into two types: antibody formation and cell-mediated responses. Our initial studies were conducted to determine whether the mediators of immunity, i.e., lymphocytes, could be identified as having any immune defects which could be related to chemical exposure and be an early signal of an impaired immune system which might interfere with the body's normal defenses. These antibody forming cells constitute the humoral part of the human immune response. The lymphocytes are divided into at least two classes or types called B cells and T cells. The B cell lymphocytes are responsible for the synthesis and secretion of antibody molecules, while the T cells are responsible for helping B cells. In addition, the T cells are also able to carry out a whole series of reactions in their own right. Generally these reactions involve tissue destruction, and since antibodies are usually not involved, they are called cell-mediated reactions. There has been recent thinking that the body's defense against tumors is a result of a tumor's having a unique antigen which is therefore recognized as foreign. The immune system constantly responds against these new antigens, and in this manner, there is an ongoing immune surveillance against tumors. Failure to react with the new antigen results in cancers, according to this theory.

Objective

1. To determine the immune competence of chemical workers with prolonged exposure to vinyl chloride monomers

2. To compare their immune response to those workers who had developed liver injury, including angiosarcoma.

Research Results

An evaluation of immunocompetence was carried on in individuals with documented chemical hepatic injury and in those without biochemical or clinical evidence of liver injury. A second comparison was conducted between workers with high versus low exposure to vinyl chloride, utilizing our cumulative exposure rank months (CERM) ranking. Tests for immunocompetence included enumeration of lymphocytes, determination of certain lymphocyte subpopulations, and response of lymphocyte to nonspecific antigens as a test of lymphocytic function.

Results of lymphocyte response to stimulation with nonspecific antigens Phytohemagglutinin (PHA), Concanavallin A (Con-A), and Pokeweed mitogen (PWM) are shown in Figure 1.

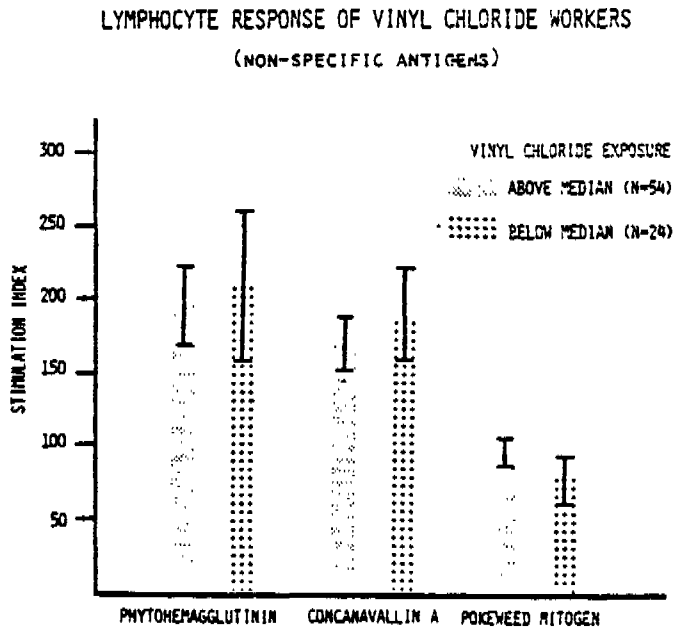

FIGURE 1

Response of the other immune parameters in high and low vinyl chloride exposure are shown in Table 1. No statistically significant difference was found between workers with high and low vinyl chloride exposure, or between unexposed invidivduals and chemical workers (Figure 2).

TABLE 1

IMMUNE PARAMETERS OF VC WORKERS
ACCORDING TO EXPOSURE

TEST	ABOVE MEDIAN EXPOSURE	BELOW MEDIAN EXPOSURE	STATISTICAL SIGNIFICANCE
STREPTOCOCCAL ANTIGEN STIMULATION	191 ±31** (N=52)	186±34 (N=24)	NONE
STREPTOLYSIN O STIMULATION	8±2 (N=53)	24±12 (N=24)	NONE
PPD STIMULATION	29±8 (N=49)	18±6 (N=23)	NONE
VARIDASE STIMULATION	29±5 (N=54)	41±12 (N=23)	NONE
CANDIDA STIMULATION	11±3 (N=53)	6±2 (N=23)	NONE
ABSOLUTE LYMPHOCYTE COUNT	2355±132 (N=52)	2316±148 (N=24)	NONE
T-CELL ROSETTES AT 4°C (ABSOLUTE)	1503±101 (N=50)	1524±132 (N=24)	NONE
T-CELL ROSETTES AT 33°C (ABSOLUTE)	1227±80 (N=50)	1260±123 (N=24)	NONE

*STIMULATION INDEX
**SEM

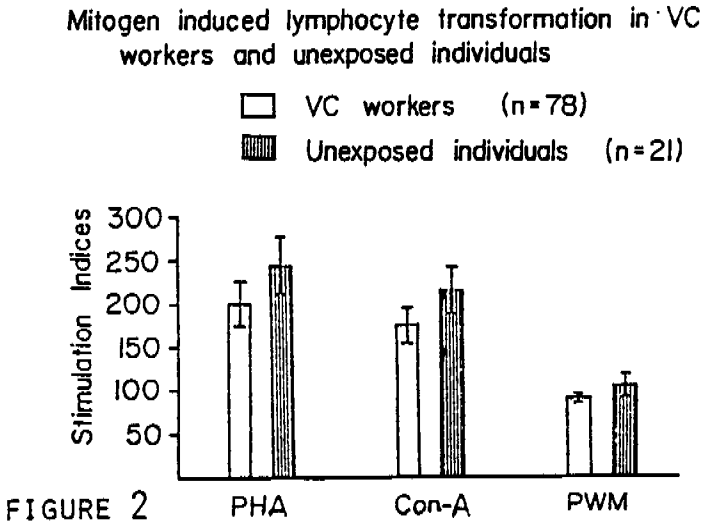

FIGURE 2

No statistical differences were seen in these same immune parameters between workers with or without liver injury (Tables 2 and 3).

TABLE 2

IMMUNE PARAMETERS OF VC WORKERS WITH AND WITHOUT LIVER DISEASE

TEST	LIVER DISEASE	NO LIVER DISEASE
PHYTOHEMAGGLUTININ STIMULATION	258*±35** (N=25)	170±35 (N=48)
CONCANAVALLIN A STIMULATION	195±36 (N=25)	162±29 (N=48)
POKEWEED MITOGEN STIMULATION	100±12 (N=25)	80±13 (N=48)
STREPTOCOCCAL ANTIGEN STIMULATION	208±42 (N=24)	181±32 (N=49)
STREPTOLYSIN O STIMULATION	8±2 (N=25)	16±6 (N=49)
PPD STIMULATION	18±6 (N=25)	30±9 (N=45)
VARIDASE STIMULATION	36±9 (N=25)	31±5 (N=50)
CANDIDA STIMULATION	14±5 (N=25)	7±1 (N=49)

*STIMULATION INDEX
**SEM

TABLE 3

IMMUNE PARAMETERS OF VC WORKERS WITH AND WITHOUT LIVER DISEASE

TEST	LIVER DISEASE	NO LIVER DISEASE
WHITE BLOOD CELL COUNT	6900±500* (N=25)	6650±380 (N=50)
LYMPHOCYTE COUNT (PERCENT)	36±2 (N=25)	38±2 (N=48)
ABSOLUTE LYMPHOCYTE COUNT	2350±200 (N=25)	2300±200 (N=48)
T-CELL ROSETTES AT 4°C (PERCENT)	67±3 (N=25)	62±2 (N=49)
T-CELL ROSETTES AT 4°C (ABSOLUTE)	1600±100 (N=25)	1500±100 (N=49)
T-CELL ROSETTES AT 33°C (PERCENT)	55±2 (N=25)	52±2 (N=47)
T-CELL ROSETTES AT 33°C (ABSOLUTE)	1300±100 (N=25)	1200±100 (N=47)

*SEM

Tables 4 and 5 show the results of these immunological parameters examined in old and young workers. Older individuals demonstrated significant differences in their total white count ($P<.01$), percentage of lymphocytes ($P<.001$), and lymphocyte reactivity to Streptolysin O ($P<.05$) and PPD (tuberculin) ($P<.05$) stimulation. We believe these findings to be age related with no relationship to the presence of liver disease or exposure to vinyl chloride. No other statistically significant differences were found between the various groups. There is no evidence from this data that immunological abnormalities result from chronic exposure to various levels of vinyl chloride.

TABLE 4

IMMUNE PARAMETERS OF VC WORKERS
OLD VERSES YOUNG

TEST	OLD	YOUNG	
PHYTOHEMAGGLUTININ STIMULATION	140*±20** (N=37)	210±32 (N=40)	
CONCANAVALLIN A STIMULATION	160±25 (N=37)	188±25 (N=40)	
POKEWEED MITOGEN STIMULATION	90±15 (N=37)	90±20 (N=40)	
STREPTOCOCCAL ANTIGEN STIMULATION	185±35 (N=38)	195±35 (N=38)	
STREPTOLYSIN O STIMULATION	5±2 (N=38)	21±7 (N=38)	P<.05
PPD STIMULATION	36±12 (N=39)	13±3 (N=35)	P<.05
VARIDASE STIMULATION	25±6 (N=38)	39±9 (N=39)	
CANDIDA STIMULATION	9±3 (N=37)	9±3 (N=39)	

*STIMULATION INDEX
**SEM

TABLE 5

IMMUNE PARAMETERS OF VC WORKERS
OLD VERSES YOUNG

TEST	OLD	YOUNG	
WHITE BLOOD CELL COUNT	7150±400* (N=38)	6150±300 (N=40)	P<.01
LYMPHOCYTE COUNT (PERCENT)	33±2 (N=36)	41±2 (N=40)	P<.001
ABSOLUTE LYMPHOCYTE COUNT	2300±200 (N=36)	2400±100 (N=40)	
T-CELL ROSETTES AT 4°C (PERCENT)	64±2 (N=36)	64±2 (N=40)	
T-CELL ROSETTES AT 4°C (ABSOLUTE)	1400±150 (N=34)	1600±100 (N=40)	
T-CELL ROSETTES AT 33°C (PERCENT)	52±2 (N=36)	53±2 (N=40)	
T-CELL ROSETTES AT 33°C (ABSOLUTE)	1100±100 (N=34)	1300±100 (N=40)	

*SEM

A2. Assessment of the Leukocyte Adherence Inhibition Test for the Detection of Angiosarcoma of the Liver

Background

Halliday et al. (1974) reported the development of a leukocyte adherence inhibition test which identified the preclinical stages of colon cancer. Similar findings were reported in patients with liver cancer--the primary hepatocellular type. The need for a more specific screening test, especially for those who might be developing angiosarcoma that was not clinically detectable, led us to assess this test in our cohort population.

Objective

1. To verify the studies of Halliday and Maluish regarding the use of the leukocyte adherence inhibition test in the identification of carcinomas of the colon and its possible adaptation to vinyl chloride-induced angiosarcoma.

Research Results

Dr. Maluish came from Australia and spent a number of days helping us develop and further validate the methodology used. Despite multiple attempts we were not able to validate the reproducibility of the leukocyte adherence inhibition test, neither in colon cancer nor in liver angiosarcoma patients. Unfortunately, we must conclude that the leukocyte inhibition adherence test is not sufficiently reproducible to be used as a reliable indicator of the presence of tumors.

A3. Assessment of Vinyl Chloride-Induced Angiosarcoma Antigen for the Detection of Latent Angiosarcoma in Exposed Workers

Background

New antigens arise on tumors formed as a response to carcinogens. Their presence on induced sarcomas was discovered by Foley (1953). This discovery in mice was verified and extended by Prehn and Maine (1957) who concluded that there were antigens particular to and specific for tumor tissue. Subsequently, evidence for specific tumor antigens was found in humans by Hellstroms, Vankey, Halliday and Maulish, Thompson, and others. The majority of the evidence suggests that tumor antigens found were distinctive for each histological type of tumor. This concept was applied to our cohort population for primary liver cancer.

Objective

1. To determine whether the human body mounts an immune reaction to developing cancer cells that can be identified by specific immune reactions with tumor-specific antigen.

Research Results

Lymphocytes (the cells responsible for immunity) were obtained from individual workers, isolated, and grown in the presence of liver reagents prepared from either normal individuals or individuals who had had angiosarcoma. A positive reaction was identified by incorporation of ^{3}H-thymidine into stimulated lymphocyte cultures as compared to unstimulated (control) cultures. Comparison of responses to a panel of tissue extracts (Table 6) by lymphocytes from vinyl chloride workers and normal non-chemical plant workers indicated that many unexposed individuals have reactivity to these tissue antigens. Lymphocyte reactivity against angiosarcoma tissue alone occurred more frequently among vinyl chloride workers (Table 7).

TABLE 6

TISSUE EXTRACT PANEL

EXTRACT	NUMBER IN PANEL
ANGIO TUMOR	3
NORMAL LIVER	3
HEPATOMA LIVER	1
ANGIO "NORMAL" LIVER*	2
NORMAL KIDNEY**	2

*"NORMAL" AREAS OF LIVER TISSUE FROM ANGIOSARCOMA LIVERS.

**OBTAINED FROM THE SAME DONORS AS NORMAL LIVERS.

TABLE 7

LYMPHOCYTE REACTIVITY TO TUMOR AND NORMAL TISSUE PANEL

	VINYL CHLORIDE WORKERS		NON-VINYL CHLORIDE WORKERS		INTERPRETATION OF RESULTS
LYMPHOCYTES REACTIVE AGAINST	N	PERCENTAGE	N	PERCENTAGE	
ANGIO LIVER ALONE	6	16	0	0	INDIVIDUALS WITH POSSIBLE SPECIFIC ANTI-TUMOR REACTIVITIES
NORMAL AND ANGIO LIVER	7	18	8	57	INDIVIDUALS WITH NONSPECIFIC REACTIVITIES MASKING ANY POSSIBLE SPECIFIC REACTIVITIES
NORMAL LIVER ALONE	5	13	1	7	INDIVIDUALS WITH NONSPECIFIC REACTIVITIES
NONREACTIVE	20	53	5	36	INDIVIDUALS WITH NO TISSUE REACTIVITIES
TOTAL	38	100	14	100	

Lymphocytic reaction to normal and angiosarcoma liver reagents were studied in workers with high and low exposure (above or below the median exposures for the plant) to vinyl chloride (Figure 3). No statistical difference was noted between the reactivities of these two groups.

LYMPHOCYTE RESPONSE OF VINYL CHLORIDE WORKERS

(TISSUE ANTIGENS)

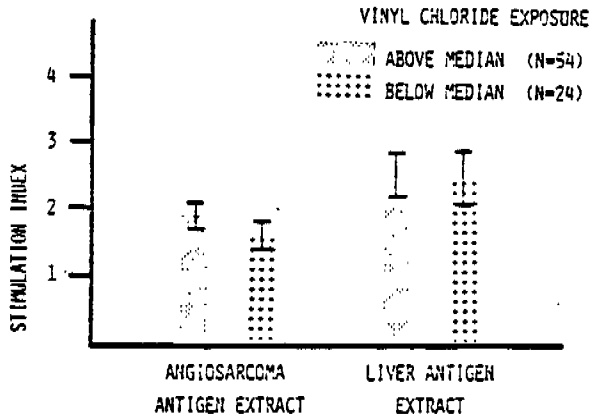

FIGURE 3

Similar studies in workers with and without liver disease also failed to show significant differences in their lymphocytic reactivity to tissue reagents (Table 8A).

TABLE 8A

LYMPHOCYTE REACTIVITY TO NORMAL AND ANGIOSARCOMA TUMOR TISSUES IN VINYL CHLORIDE WORKERS WITH AND WITHOUT LIVER DISEASE

	LIVER	
	NORMAL TISSUE	TUMOR TISSUE
LIVER DISEASE	2.4 ± 0.3*	1.5 ± 0.2
NORMAL	2.6 ± 0.4	1.8 ± 0.2

*STIMULATION INDEX ± SEM

On the chance that non-specific reactions might be obscuring the specific reactions, workers with vinyl chloride-suspected disease (working in a non-vinyl chloride environment--Pallet Plant) were compared to workers without known liver disease (working at the Main Plant).

TABLE 8B

IN VITRO LYMPHOCYTE STIMULATION BY ANGIOSARCOMATOUS LIVER EXTRACT

SUBJECTS	NUMBER TESTED	NUMBER OF SUBJECTS REACTIVE AGAINST:		
		NORMAL LIVER ONLY	NORMAL & ANGIO	ANGIO ONLY
NON-PALLET PLANT WORKERS	16	1	1	1
PALLET PLANT WORKERS	18	1	0	2*
TOTAL	34	2	1	3

*ONE INDIVIDUAL HAD ANGIOSARCOMA

At first, this appeared to be a specific reaction to the angiosarcoma tumor extract, i.e., negative reactions to normal liver with positive reactions to tumor antigen. Initially, this type of reactivity occurred only among the vinyl chloride workers. Subsequent studies among additional nonchemical workers demonstrated similar reactivities to these liver reagents. Although these results were provocative, the lack of further occurrences of angiosarcoma in our worker population prevented us from further characterizing this (these) possible angiosarcoma antigen(s) and having the opporunity to verify these observations.

A4. Study of Human Leukocyte Antigen (HLA) Frequencies in Chemically-Exposed Workers

Background

The major histocompatibility complex of the immune system, HLA, is composed of multiallelic genes located on one region of chromosome 6 in the human and on chromosome 17 in the mouse. The genes in this region control a wide variety of surface antigens and lymphoid functions. This major histocompatibility complex is intimately related to the understanding of transplantations, graft versus host reactions, interactions of B and T Cells, genetic control of immune responses and several other phenomena in the immunological system. Certain genetic traits appear to be controlled in animals by the genes of this complex. These include transplantation antigens, cell-mediated lympholysis,

target antigens, immune response, tumor virus susceptibility, liver cyclic adenosine monophosphate levels, hybrid resistance, and T-cell: B-cell interactions. HLA antigens are found on almost all cells of the body except red blood cells. The major histocompatibility complexes in man are identified by letters (A, B, C, D) and numbers (A10, B12, C3). An increased incidence of certain HLA antigens had been shown to be associated with susceptibility to certain diseases. Mulvihill (1976) proposed that screening for these genetic markers to identify abnormal genotypes in potential employees should be done so that individuals who might be predisposed to neoplasia after occupational exposure would be separated from those from normal genotypes. The first occupational disease correlation reported was HLA-B27 antigen which was found with increased frequency in workers suspected of having occupational asbestosis. The possibility that the histocompatible complex could be used to identify individuals at increased risk of developing chemical injury led to its study in vinyl chloride workers.

Objective

1. To determine the HLA frequency in vinyl chloride workers
2. To compare the HLA frequency in vinyl chloride workers with and without liver disease.

   In order to deal with the statistical problem of multiple comparisons due to the large number of antigens to be studied, potential markers for liver disease were identified for study: A-9, B-15 and B-17.

Research Results

HLA determinations were done using the Terasaki microdroplet lymphocyte cytotoxicity test. HLA typing was performed on 538 of our chemical cohort population. Tissue typing included 11 HLA-A antigens, 15 HLA-B antigens and 4 HLA-C antigens. Frequency distributions were determined in our "standard" (healthy population) and compared with the "normal" groups studied by Scott et al. (1977) and the World Health Organization. The comparison of these frequencies are found in Tables 9 and 10.

A preliminary analysis of these HLA frequencies included the comparison of a subcohort population consisting of individuals identified through the medical screening program as having liver disease. These individuals had been transferred from the main chemical plant to an ancillary plant making wooden shipping pallets, referred to subsequently as "Pallet Plant Cohort".

As seen in Table 10 this cohort had increased frequency of HLA-B15 (12 percent-normal population versus 28 percent-Pallet Plant Cohort).

TABLE 9

HLA-A FREQUENCIES

HLA-A ANTIGENS	HEALTHY CONTROLS	WHO 1974 WORKSHOP	PALLET PLANT(BFG)	NON PALLET PLANT(BFG)
A1	34	32	46	30
A2	51	49	57	52
A3	23	22	14	21
A9	16	17	11	22
A10	7	13	18	6
A11	13	8	7	11
A28	7	11	18	12
A29	9	7	0	8
AW30	ND**	5	4	6
AW31	ND	7	4	6
AW32	ND	8	7	7
Blank	ND	ND	14	19
N	900	503	28	519
Total Frequency	160%	170%	200%	200%

**NOT DONE

TABLE 10

HLA-B FREQUENCIES

HLA-B ANTIGENS	HEALTHY CONTROLS	WHO 1975 WORKSHOP	PALLET PLANT/BFG	NON PALLET PLANT/BFG
B5	10	11	11	9
B7	31	23	18	28
B8	27	20	21	20
B12	30	24	21	31
B13	3	6	0	5
B14	5	11	11	8
B15	10	7	29	12
BW16	ND	12	7	4
B17	6	7	14	12
B18	8	9	4	6
BW21	ND	4	4	4
BW22	2	5	7	5
B27	7	8	7	11
BW35	5	17	11	17
B40	11	12	18	16
Blank	ND	ND	18	13
N	903	503	28	519
Total Frequency	155%	176%	201%	201%

Table 11 illustrates the frequency of HLA-B15 among the entire worker cohort at the main chemical plant. This cohort was subdivided into those with liver disease versus normal individuals based on medical screening studies and liver biopsy. HLA-B15 occurred in 13 percent of the normal group versus 17 percent of the liver diseased group.

TABLE 11

DISTRIBUTION OF HLA-B15 ANTIGEN AMONG
CHEMICAL WORKERS WITH AND WITHOUT LIVER DISEASE
(MAIN PLANT COHORT)
CLINICAL DIAGNOSIS

N=509

B15	NORMAL	LIVER DISEASE
+	64	3
-	427	15
TOTAL	491	18
(%)	(13)	(17)

To determine if this increased occurrence of HLA-B15 was due to chemically-related liver injury and not due to incidental (non-occupational) liver disease, both the pallet plant and the main plant cohorts with liver disease were subclassified into those with chemical liver injury (CLI), and non-chemical liver disease (LD). The frequency of HLA-B15 in these cohorts is shown in Tables 12 and 13.

Table 14 illustrates the occurrence of HLA-B15 in the entire plant cohort subclassified into those without liver disease (NORMAL), those with liver disease, all types (BOTH). Those with liver disease are further subclassified into those with chemical liver injury (CLI) and those with liver disease, non-chemical in origin (LD).

TABLE 12

DISTRIBUTION OF HLA-B15 ANTIGEN AMONG WORKERS WITH AND WITHOUT CHEMICAL RELATED LIVER DISEASE (PALLET PLANT COHORT)

CLINICAL DIAGNOSIS

N=29

	NORMAL	CLI	LD
+ B15	1	4	3
-	3	9	9
TOTAL	4	13	12
(%)	(25)	(31)	(25)

TABLE 13

DISTRIBUTION OF HLA-B15 ANTIGEN AMONG WORKERS WITH AND WITHOUT CHEMICAL RELATED LIVER DISEASE (MAIN PLANT COHORT)

CLINICAL DIAGNOSIS

N=526

	NORMAL	CLI	LD
+ B15	65	0	3
-	430	2	13
TOTAL	495	15	16
(%)	(15)	(0)	(19)

TABLE 14

OCCURRENCE OF HLA-B15 AMONG CHEMICAL WORKERS WITH AND WITHOUT CHEMICAL LIVER INJURY

CLINICAL DIAGNOSIS

N=538

	NORMAL	CLI	LD	BOTH
+ B15	65	4	6	10
-	430	11	22	33
TOTAL	495	15	28	43
(%)	(13)	(27)	(21)	(23)

A5. Identification of the Endothelial Cell as the Cell of Origin for Vinyl Chloride-Induced Angiosarcoma of the Liver

Background

A search for evidence of vinyl chloride-induced tumor antigen has led to the finding that these tumors have an increased concentration of antigenic coagulation Factor VIII. Factor VIII is a known marker for the vascular lining cells of the endothelial type. Further experiments in animals indicated that the normal endothelial cell found lining the liver sinusoids has little if any Factor VIII fluorescence. Endothelial cells found lining larger vessels on the other hand demonstrated a striking fluorescence, as did experimentally transplanted mouse angiosarcomas. Conversely, mouse hepatic Kupffer cells, a second type of hepatic lining cell distinguished in histological cross section by engorgement with carbon particles, failed to demonstrate positive fluorescence. An increase in antigenic Factor VIII in hepatic angiosarcoma endothelial cells as compared to the normal, led us to believe that these cells have increased production or storage capacity for antigenic Factor VIII.

Objective

1. To study the Factor VIII fluorescence in human angiosarcomas, primary hepatocellular carcinomas, and normal livers.

Research Results

Examination of angiosarcoma liver demonstrated a significant fluorescence due to Factor VIII content in the malignant cells of the liver sinusoids. An intermittent pattern of fluorescence of the sinusoidal lining cells was seen. These cells were histologically identified as angiosarcomatous on hematoxylin and eosin (H & E) staining of adjacent tissue. This type fluorescence was not seen in individuals with primary hepatocellular carinomas and a different pattern was seen in normal livers. These cells and their fluorescent pattern are illustrated in Figures 4 and 5. Figure 4 illustrates the H & E stained cells and Figure 5 their Factor VIII fluorescence.

FIGURE 4

FIGURE 5

Relevance to Industry

These rather extensive, detailed studies of a well-defined human population provide very valuable data regarding the use of immunological screening tests for the detection and identification of chemically-related injury.

(1) There appears to be no significant evidence that the immunodepression or change in the antibody responding components or the cell-mediated responding components of the human immune system is affected by vinyl chloride exposure. Therefore, studying immunological parameters of type B or T cells will not provide any useful information in the early stages of disease even with excessive exposure.

(2) The use of tumor antigens for the detection of developing angiosarcoma, although a provocative possibility, is far too non-specific in its present stage of development to provide any reasonably useful means of detecting early cancer development. Until there is better evidence that tumor antigens provide sufficient specificity to prevent cross reactivity with other tissue materials, this approach will not appear to be a clinically useful one.

(3) The present data suggest that the HLA-B15 antigen may reflect an increased susceptibility to liver injury, especially of chemically-induced origin. Confirmation of these findings and the determination of the effectiveness of this marker in screening those with increased susceptibility to chemical liver injury must yet be determined by prospective study.

(4) The leukocyte adherence inhibition test, unfortunately, was not adequate in its technical development nor sufficiently reproducible to be useful as a screening test for either colon cancer or liver angiosarcoma.

All of the above immunological studies, with the possible exception of HLA marker, have provided little supportive evidence that chemical injury interferes with human immunocompetence as determined by our present methods of study. Therefore, studies of the immunological system for screening purposes appear to be of little, if any, value in the early identification of chemical injury or disease.

(5) The identification of Factor VIII production by human and animal angiosarcoma tumors provides further evidence for the need to develp screening methods to detect endothelial rather than hepatocytic cell injury or dysfunction since these are the cells of origin that malignantly transform when exposed to vinyl chloride and other agents such as arsenic.

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PROGRAM B

STUDY OF HEPATIC BIOCHEMICAL AND ENZYMATIC SYSTEMS FOR THE IDENTIFICATION OF VINYL CHLORIDE AND OTHER CHEMICAL INJURY IN CANCER DEVELOPMENT: ANIMAL AND HUMAN STUDIES; Investigators - J.T. Du, C.H. Tamburro and M.T. Tseng

ANIMAL STUDIES:

B1. Characterization Of Hepatic Enzyme Changes In Rats With Prolonged Vinyl Chloride Exposure: Decreased Glucose-6-Phosphatase Activity

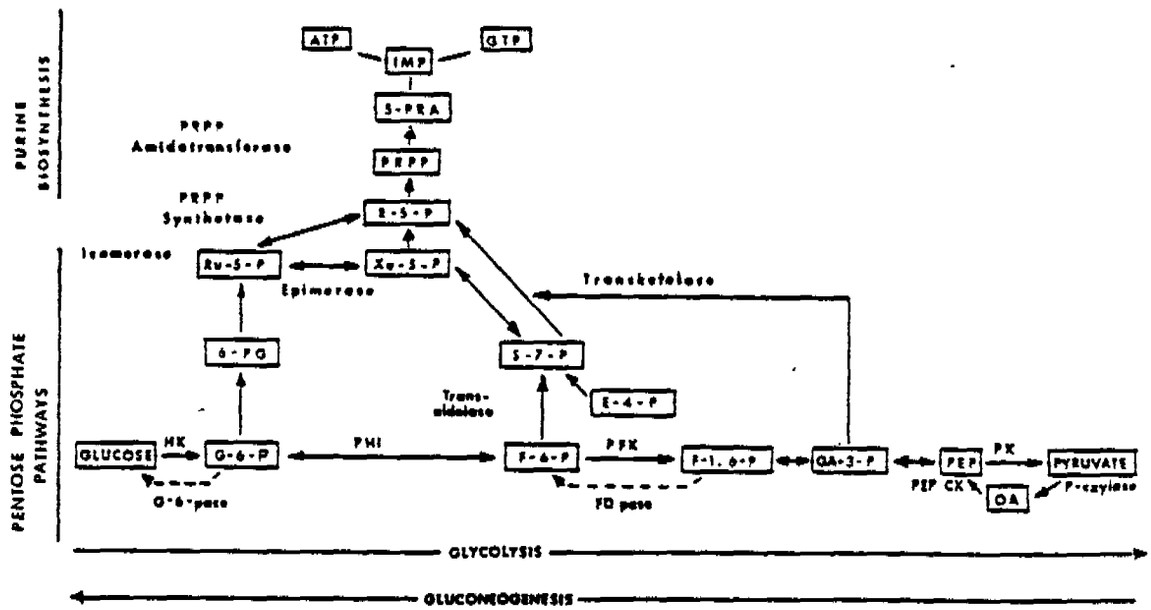
B2. Morphological Alterations In Livers Of Rats Exposed To Vinyl Chloride

Background

Vinyl chloride has been shown to induce an identical malignancy in both animals and man. Vinyl chloride-induced liver cancer in animals, therefore, provided an excellent model for the study of chemically-induced carcinogenesis. The rat model provides the opportunity to further elucidate the mechanism and the pathogenesis of this particular chemical agent, whose major oxidation occurs in the hepatocytes (primary liver cells) while inducing a malignant transformation in an adjacent, different cell type (endothelial lining cell of the sinusoids). Vinyl chloride had been shown to cause angiosarcoma and primary hepatocellular carcinoma in laboratory animals (Maltoni, 1975, 1976), and angiosarcoma in man (Creech and Johnson, 1974). However, little was known regarding the biochemical changes that vinyl chloride mediated to induce hepatocellular injury and subsequent malignant transformation of the mesenchymal cells.

Enzymatic alterations in primary liver parenchymal cell cancers (hepatocellular carcinoma, hepatoma) have been studied extensively. Weber and Convery (1966) and Weber and Lea (1967) proposed that there was a biochemical pattern which could be related to the biological behavior of neoplastic cells. Using a spectrum of hepatomas with different growth rates, a basis for underlying neoplastic cell transformation was proposed as being related to the cell's progressive alterations in the molecular pattern which led to progressive increases in the growth rate of the tumors. Figure 1 outlines the pathways.

FIGURE 1 WEBER'S MOLECULAR CORRELATION CONCEPT OF NEOPLASIA



Key enzymes of glycolysis were shown to increase while key enzymes in gluconeogenesis decreased with tumor growth. In addition, the pentose phosphate biosynthetic pathway, glucose-6-phosphate dehydrogenase (Weber and Morris, 1963) and transaldolase (Heinrich et al., 1974) were shown to be increased in all hepatoma studies. Furthermore, the activity of phosphoribosylpyrophosphate (PRPP) synthetase and glutamine PRPP aminotransferase, the first two enzymes channeling ribose-5-phosphate into purine, DNA and RNA synthesis were also increased. The PRPP sythetase was increased in rapidly growing hepatomas and the transferase in all the hepatomas irrespective of growth rates (Weber et al., 1975). Decreased enzyme activity was observed at an early stage of carcinogenesis before the morphological signs of a tumor appeared in the liver, and the activity of enzymes dependent upon the rate of hepatoma proliferation correlated with progressive malignant transformation.

These findings and the lack of any long-term sequential studies of animals exposed to vinyl chloride directed us to our first three objectives.

Objectives

1. To determine the sequential hepatic enzymatic changes in animals chronically exposed to vinyl chloride and their correlation to conventional clinical tests of liver function.

2. To determine if these sequential enzymatic patterns, especially with regard to carbohydrate metabolism and nucleic acid synthesis, would correlate with vinyl chloride-induced liver injury and ultimate malignant transformation.

3. To correlate the sequential, morphological changes with these enzymatic findings.

Research Results

1. Characterization of Hepatic Enzyme Changes

Sprague-Dawley adult male rats were chronically exposed to vinyl chloride. The initial studies characterized the metabolic alteration in the liver by studying various organelle enzyme markers which included glucose-6-phosphatase (microsomal), cytochrome oxidase (mitochondrial) and glucose-6-phosphatase dehydrogenase (cytosol). In addition, standard serum biochemical clinical liver studies of the liver (liver "function" tests) were performed including aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), alkaline phosphatase, lactic acid dehydrogenase, total protein, and bilirubin. Additional studies of subcellular enzymes related to nucleic acid synthesis--glutamine PRPP aminotransferase, and glycolysis--phosphofructokinase were done. In vivo protein synthesis was assessed by ^{3}H-leucine incorporation.

The results of these studies illustrated that rats exposed to high chronic doses of vinyl chloride had a 25% lower activity of glucose-6-phosphatase, a key enzyme for gluconeogenesis after about 71 hours of exposure and a 50-100% increase in glucose-6-phosphate dehydrogenase, a rate limiting enzyme in the pentose pathway, after 84 hours of exposure.

These studies demonstrated that (a) metabolic alterations in the liver cell are induced by vinyl chloride exposure, (b) gluconeogenesis is altered as reflected by a lower glucose-6-phosphatase and a higher glucose-6-phosphatase dehydrogenase activity, and (c) that these changes occur in a fashion similar to the lower gluconeogenic and higher pentose shunt activity of hepatomas. Although this could result in an enhanced production of PRPP and ultimately more nucleic acid synthesis, our studies of PRPP did not document this as a subsequent finding. These enzymatic changes, although similar to the biochemical changes seen in rapidly growing primary hepatocellular carcinomas, most likely reflect liver cell adaptation to vinyl chloride exposure but do not appear to significantly increase DNA synthesis. In addition, these changes were sufficiently limited so as not to be reflected by circulating enzyme levels traditionally used for the detection of clinical hepatocellular injury (Figure 2).

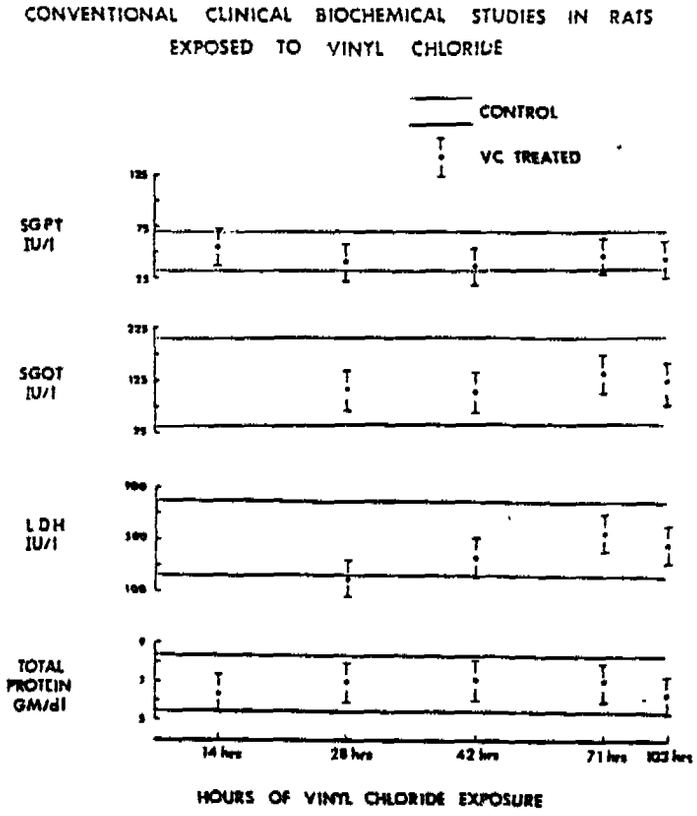


FIGURE 2

2. Morphological Alterations: Light and Electron Microscopic Assessment

Sequential morphological assessment was performed in both of the chronic experiments utilizing light and electron microscopy.

The enzymatic findings discussed in B1 and subsequently in B3 were associated with light microscopic findings which showed a marked increase in liver cell polyploidy, double nucleated cells and areas of focal hepatocellular hyperplasia. There was no evidence of hepatocellular injury, necrosis, or increased fibrosis. The morphological changes seen, however, reflect an increased liver cell regeneration which was not measurable by the techniques used to measure DNA synthesis in our experiments.

The electron microscopic changes demonstrated a proliferation of the smooth endoplasmic reticulum without evidences of changes in the hepatocytic cell, cell membrane, nucleic acid or rough endoplasmic reticulum structures.

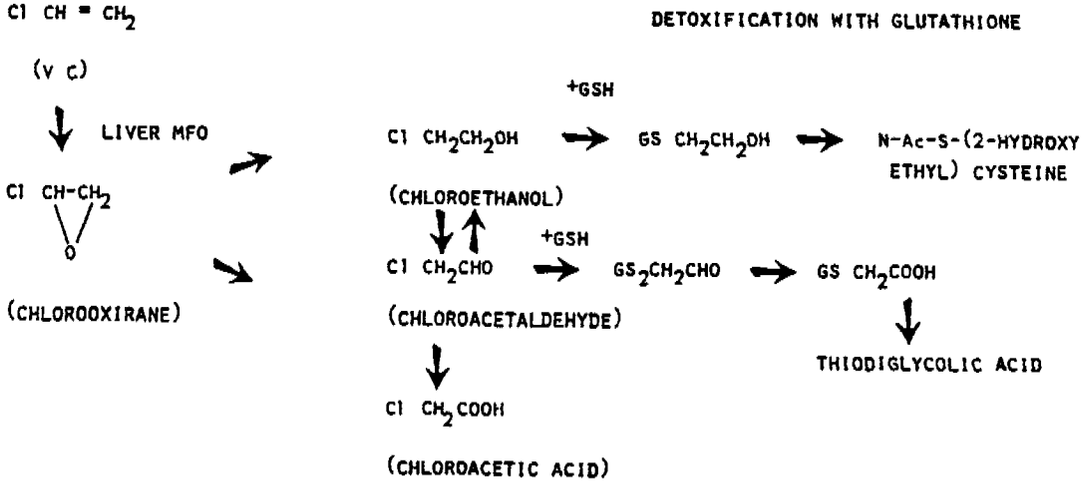
There was no evidence of increased collagen deposition in the space of Disse which has been reported by others during terminal or final stages of angiosarcoma development in animals.

B3. Alterations in the Oxidizing and Detoxifying System of the Rat Liver After Prolonged Exposure to Vinyl Chloride.

Background

Vinyl chloride metabolism had been shown to be blocked at low dosage levels (50 ppm) by inhibiting the alcohol dehydrogenase system and at higher levels (200 ppm) by metabolism via the microsomal mixed function oxidase (MFO) system (Hefner et al., 1975, Bolt et al., 1975). Vinyl chloride's major metabolites, chlorooxirane and chloroacetaldehyde, have been identified as probable ultimate carcinogens (Barbin et al, 1975; Van Duuren, 1975; and Jaeger et al., 1974). Further studies (Elmore et al., 1976; Malaveille et al., 1975; McCann et al., 1975) demonstrated the mutagenic activity of chlorooxirane and chloroacetaldehyde on microorganisms and the nonmutagenicity of chloroethanol and chloroacetic acid, other metabolites of vinyl chloride.

Detoxification of these metabolites was reflected by a reduction in non-protein sulfhydryl content of the liver (Hefner, 1975) and tracer studies showing major urinary metabolites of vinyl chloride to be N-acetyl-S-hydroxyethylcysteine and thiodiglycolic acid (Watanabe et al., 1976). The proposed metabolic fate of vinyl chloride is illustrated:



In these experiments, the enzymes studied related to vinyl chloride oxidation, included P-450, NADPH-cytochrome-C-reductase and mixed function oxidase. Those enzymes related to detoxification include non-protein sulfhydryl content (NPSC), glutathione content (GSH), glutathione reductase (GR), glutathione-epoxide-S-transferase (GEST, glutathione-E-transferase), and glutathione-aralkyl-S-transferase (GAST, glutathione A&B transferases).

Objective

1. To determine the sequential changes of key hepatic oxidizing and detoxifying enzymes with prolonged vinyl chloride exposure.

Research Results

The oxidizing and detoxifying capability of rat liver cells during prolonged exposure to chronic high doses of vinyl chloride demonstrated a 25-50% higher activity of glutathione reductase, the enzyme which regenerates reduced glutathione to detoxify the metabolites of vinyl chloride. Although our acute and single vinyl chloride exposure studies demonstrated a decrease in the nonprotein sulfhydryl content (48%) similar to that reported by Hefner (1975), repeated or chronic exposure to higher levels showed a progressive increase which was statistically significant after 70 hours of exposure (Figure 3).

Glutathione-epoxide-S-transferase (GEST) and glutathione-aralkyl-S-transferase (GAST), the enzymes capable of conjugating the toxic vinyl chloride metabolites, were significantly elevated following vinyl chloride exposure; glutathione reductase (GR), the enzyme to regenerate glutathione, was also elevated significantly following vinyl chloride exposure (Figure 4).

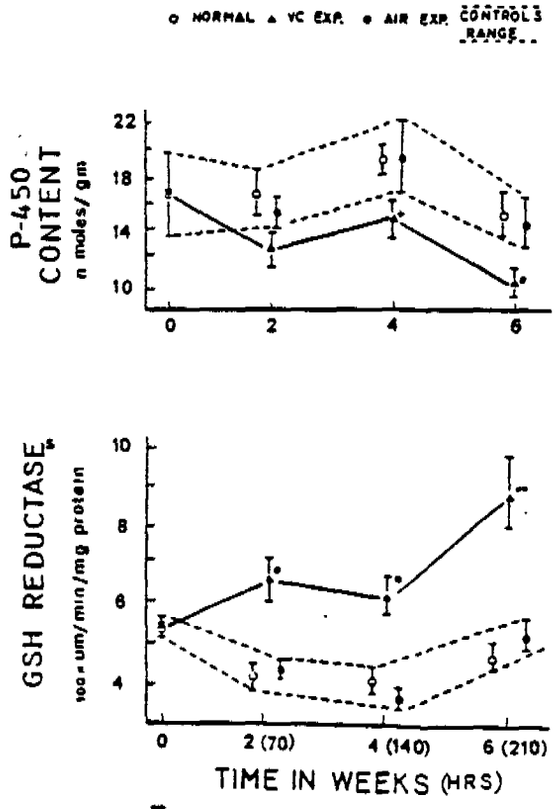


FIGURE 3

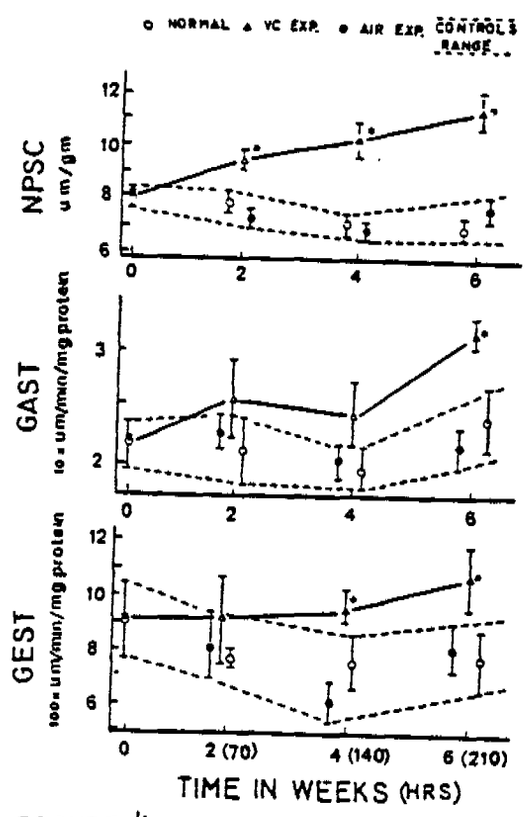


FIGURE 4

The oxidizing capability as reflected by cytochrome P-450, the major protein involved in vinyl chloride metabolism, however, was reduced significantly only after prolonged vinyl chloride exposure (Figure 3), supporting reports in vivo (Reynolds et al., 1975) and in vitro (Guengerich and Strickland, 1977; Ivanetich et al., 1977) that vinyl chloride metabolites destroyed P-450. No differences were found in the conventional clinical serum biochemical tests or in other cellular enzyme studies (cytochrome-C oxidase, NADPH-cytochrome-C-reductase and mixed function oxidase).

These changes seem to reflect early hepatic cell adaptation to chronic vinyl chloride exposure by increasing their capacity for detoxification and decreasing their capability to further metabolize vinyl chloride into its toxic intermediates--both provide a means of natural defense to a toxic substance. This entire detoxifying sequence is shown in Figure 5 as a composite of the detoxifying enzyme changes over a prolonged period of exposure.

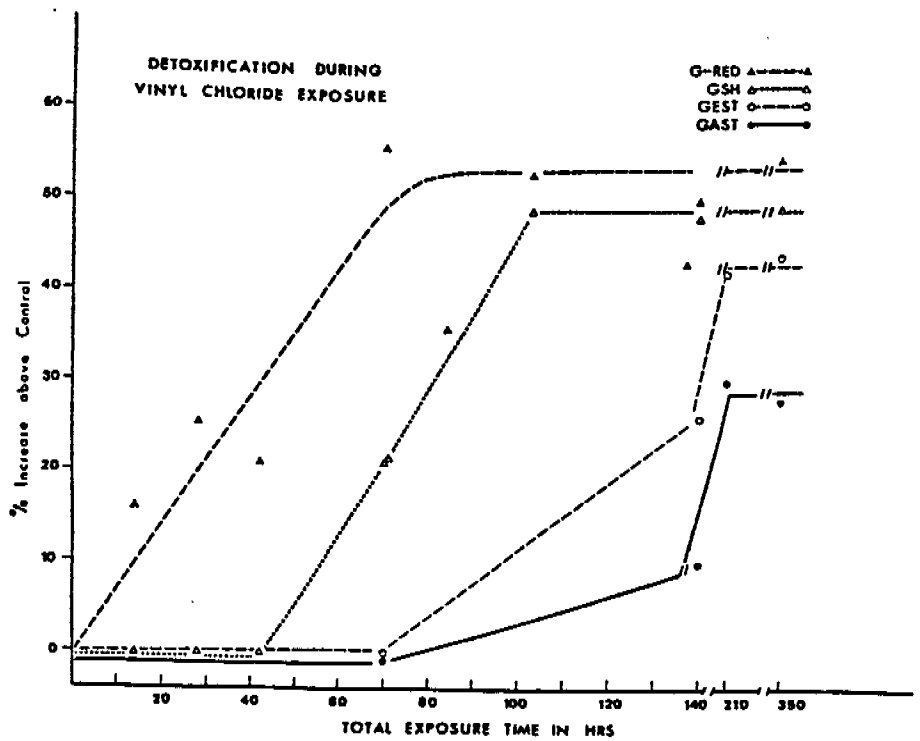


FIGURE 5

It is interesting to note that there is an earlier increase in the GEST transferase than the GAST transferases with prolonged exposure. This may reflect an earlier (in time) need for adaptation to excessive production of the epoxide and subsequently the accumulation of the aldehyde metabolite. Whether this reflects the maximum adaptability as indicated by a leveling off of enzyme after 200 hours, or whether these changes reflect an adaptation to

prevent cellular injury as well as prevent primary hepatocellular malignant transformation would require even longer sequential studies for confirmation; these were beyond our time constraints.

B4. Oxidizing and Detoxifying Ability of Liver Mesenchymal or Parenchymal Cells in the Metabolism of Xenobiotics.

Background

Even though the malignant transformation induced by vinyl chloride occurred in the mesenchymal cells of the liver (endothelial cells), all metabolic studies up to this time had been directed at the liver as a whole or to the parenchymal cell--the hepatocyte. There had been no differentiation between the oxidizing capability of the hepatocytes that demonstrated injury and the endothelial cells which were malignantly transformed. Little was also known regarding the variable ability of these two cell types in the removal of the ultimate carcinogens or active metabolites. Therefore, our next set of studies was directed toward the ability of two different cell types to metabolize vinyl chloride.

Objective

1. To study the comparative ability of the hepatocytes versus the mesenchymal cells to metabolize vinyl chloride and determine the detoxifying capability of the various liver cell types.

Research Results

Enzymatic studies of the detoxifying activities of the subcellular fraction of liver cells, both hepatocytic and mesenchymal included the study of GEST, GAST, GR, as well as P-450 and mixed function oxidase capability of the various cell fractions. The effectiveness of the isolation of the hepatocytes and mesenchymal cells was confirmed by studying pyruvate kinase activity.

Mesenchymal cells were shown to have about half the GEST activity, 7% of the GAST activity, less than half of the microsomal function oxidase activity, and 73% of the GR capability compared to the hepatocytes. The activity per million cells indicated that the nonhepatocytes had about 1/70 MFO activity, 1/65 GEST activity, 1/500 GAST activity, and 1/45 GR activity. These results demonstrate that the nonhepatocyte has significantly less capability in both the activation and detoxification of vinyl chloride and other xenobiotics. The data supports the hypothesis that the endothelial cells have a more limited capability to detoxify vinyl chloride metabolites and that this limited capability provides a greater possibility for DNA injury and subsequent malignant transformation. Again, unfortunately much longer

experimental studies would be needed to eventually validate this hypothesis (Du et al., preprint attached in Appendix).

Relevance to Industry

(1) These chronic sequential studies are highly relevant to the chemical industry's environmental health problems. The sequential, biochemical changes that occur in the carbohydrate metabolism of the liver hepatocytes reflect adaptation of the liver cell to chemical exposure. This is further reflected by the histological changes which show polyploidy, double nucleated cells and areas of focal hyperplasia. As the clinical studies in this report will subsequently show, these changes are characteristic of chemical injury but do not necessarily reflect premalignancy. This would be consistent with the enzymatic studies failing to demonstrate any evidence of increased nucleic acid synthesis which is seen in the premalignant hepatoma studies of Weber and others. The important fact is that these changes cannot be detected by the standard federally-required biochemical studies, because these are directed solely at the hepatocyte. The hepatocyte may become injured but it does not usually malignantly transform.

(2) The oxidative and detoxifying results of this study demonstrate that the hepatocyte progressively adapts its ability to remove and detoxify the active metabolites. This occurs without evidences of hepatocellular injury, at least up to what would be equivalent to 6-20% of the working years of a chemical worker. The studies also emphasize the fact that at very high exposures, the detoxifying system is adaptive and the oxidizing system's capacity is reduced, thereby providing a relative defense against continued formation of toxic metabolites.

(3) The massive ability of the hepatocyte to oxidize as well as detoxify the active metabolites of vinyl chloride can well explain why there is no hepatocellular carcinoma development in humans with vinyl chloride exposure. A markedly reduced ability of the nonhepatocyte to detoxify the metabolites while still having the capacity to oxidize vinyl chloride into its active metabolites would support the higher probability of this cell becoming malignantly transformed. Since all clinical screening studies are directed toward the hepatocyte, these animal studies emphasize the insensitiveness of clinical biochemical studies to correctly identify early injury in the more susceptible cell, i.e., endothelial cell. More sensitive indicators of nonhepatocytic cell injury or dysfunction are needed for the detection of early chemical injury. These indicators must take into account the function of both types of liver cells.

(4) The morphological findings described in the animal experiments confirm earlier observations regarding the earliest histological manifestations of vinyl chloride injury. They support our clinical observations that these lesions can be used to identify chemical injury. Program D, on the morphormetric and histological analysis details the clinical results found in humans.

HUMAN STUDIES:

B5. Effectiveness of Indocyanine Green Clearance in the Detection of Liver Injury.

Background

The increasing concern over the potential health hazard of synthetic chemicals in the occupational environment has led the government to require, and many industries to establish, medical laboratory screening programs for their employees. The primary objectives of these screening programs is to identify potential work-related disease, so that intervention may reduce disability, morbidity, and mortality. Most screening studies are directed toward the detection of abnormalities in certain body systems. The specific tests used are most often selected on the basis of medical experience in symptomatic or hospitalized populations. Prior experiences utilizing non-specific, multi-phasic health screening have not proven to be effective in this area. The effectiveness of standard medical testing used predominately in asymptomatic individuals with subclinical low-grade disease--both work and non-work related--had not been determined. This study assessed the effectiveness of using clearance studies of anionic dyes in the detection of liver injury in such individuals.

Objective

1. To determine the effectiveness of ICG clearances in detecting hepatic injury.

2. To compare ICG sensitivity and specificity to standard clinical biochemical screening in the detection of liver injury.

Research Results

The study group (cohort) consisted of 969 male employees who were tested between June 1, 1976 and May 31, 1977 with standard biochemical studies of the liver which included alanine aminotransferase (ALT/SGPT), aspartic aminotransferase (AST/SGOT), gamma glutamyl transpeptidase (GGTP), alkaline phosphatase (AP), and total bilirubin (TB). In addition, these employees were also screened by ICG clearances at the 0.5, 2.5, and 5.0 mg/kg doses, and by two more specific liver enzyme tests (isocitric dehydrogenase [ICD] and sorbitol dehydrogenase [SDH]). This main cohort was divided into a "standard" and a "non-standard" population for purposes of analysis. The division was based upon the best medical opinion. The "standard" group demonstrated no clinical evidence of significant medical disease--occupational or non-occupational in origin, and "non-standard" included all the others.

A subcohort of 120 individuals who had undergone liver biopsies for medical reasons, whether related to work or not, were also studied. This subcohort population was divided into those individuals with and without

histological evidence of liver injury, and the former further subdivided into those with and without histological characteristics of chemical injury (see Program D2).

All employees had individual work histories and rank-ordered exposure indices for 22 different chemicals used within the work place.

The GGTP provided the highest positive predictive value as a screening test for non-standard individuals (population with medical disorder). It also provided the highest sensitivity and specificity sum among the 5 most frequently abnormal tests (AST/SGOT, ALT/SGPT, AP, ICG); 2 other tests also had high predictive values--the indirect bilirubin, due to the high number of congenital indirect hyperbilirubinemic individuals, and the triglyceride levels, most likely reflecting differences in age, weight, and diabetic status in the non-standard population.

The ICG clearance (0.5 mg/kg) espressed as mean t 1/2 in minutes (min.) demonstrated differences not only between standard and non-standard population but also differences between those with high and low mean exposure to vinyl chloride (Table 1).

TABLE 1

INDOCYANINE CLEARANCE IN A COHORT INDUSTRIAL POPULATION

SUBGROUP	NO.	MEAN MIN.	90%	100%
STANDARD	662	2.9	2.0-3.8	1.8-4.8
LOW VC EXPOSURE	453	2.9	1.8-4.0	1.7-4.3
NON-STANDARD	257	3.4	2.1-5.6	2.0-6.6
HIGH VC EXPOSURE	466	3.1	2.1-4.4	1.9-4.8

The biochemical tests which best correlated with the presence of hepatic disease in the chemical workers with clinical evidence of liver disease were ALT (SGPT), GGTP, alkaline phosphatase, and ICG clearance (i.e. these were the tests with the highest sensitivity in detecting latent liver disease). Sensitivity alone, however, is not an adequate indicator of a test's screening value, especially when used in asymptomatic individuals. More appropriate evaluation of the screening ability of the tests requires that both sensitivity and specificity be determined, i.e., identify the diseased individuals and correctly exclude the non-diseased individuals. The sensitivity and

specificity of the 4 most sensitive biochemical screening tests and the ICG clearances (all doses) demonstrated that GGTP although the most sensitive is the least specific; AP has the highest specificity but the lowest sensitivity. ICG clearances, even at the low dose, clearly remain the best test, i.e., in combined sensitivity and specificity for screening and detecting individuals with subclinical liver disease (Table 2 a,b,c).

TABLE 2

A

SENSITIVITY AND SPECIFICITY
OF INDOCYANINE GREEN CLEARANCE

NO. PATIENTS 1500

		ICG 0.5 mg/kg (+)	ICG 0.5 mg/kg (-)	TOTAL
BEST MEDICAL ASSESSMENT	(+)	203	140	516
	(-)	88	1923	2011
				2439

SENSITIVITY: 55.8%
SPECIFICITY: 95.6%

B

SENSITIVITY AND SPECIFICITY
OF INDOCYANINE GREEN CLEARANCE

		ICG 2.5 mg/kg (+)	ICG 2.5 mg/kg (-)	TOTAL
BEST MEDICAL ASSESSMENT	(+)	45	28	73
	(-)	14	396	410
				483

SENSITIVITY: 45/73 = 61.6%
SPECIFICITY: 396/410 = 96.5%

C

SENSITIVITY AND SPECIFICITY
OF INDOCYANINE GREEN CLEARANCE

		ICG 5.0 mg/kg (+)	ICG 5.0 mg/kg (-)	TOTAL
BEST MEDICAL ASSESSMENT	(+)	78	6	84
	(-)	4	38	42
				126

SENSITIVITY: 78/84 = 92.9%
SPECIFICITY: 38/42 = 90.5%

In addition ICG clearance demonstrates excellent correlation with the histological presence of liver disease and liver cancer (Figure 6).

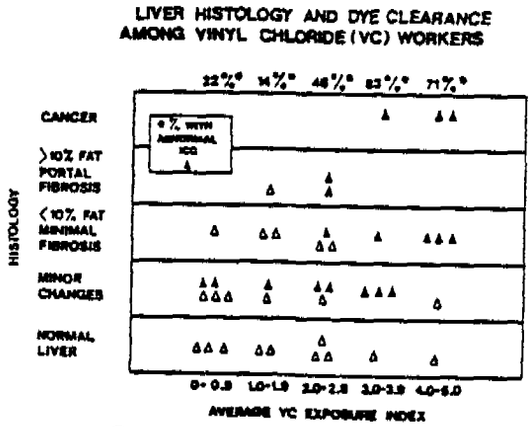


FIGURE 6

The subcohort biopsied population was additionally studied with regard to the sensitivity and specificity of the screening tests, the specific histological interpretation of the liver biopsy, and the relationship to vinyl chloride exposure. All biopsied individuals were subclassified into three groups: those with histological evidence consistent with chemical liver

injury, those with histological evidence of liver disease without evidence of chemical injury, and those with normal liver biopsies. Each group was then evaluated regarding the sensitivity and specificity of the biochemical tests and their total exposure to vinyl chloride based on a rank ordered scale.

Analysis of the subcohort biopsied group's work histories demonstrated that those individuals with chemical liver injury had the highest average rating for vinyl chloride exposure and were best identified by screening with ICG clearance tests.

B6. The Assessment of Bile Acids Vs. Indocyanine Green (ICG) Clearances in the Detection of Liver Injury in a Chemically-Exposed Worker Population

Background

Since highly sensitive and specific screening tests for the detection of latent hepatic injury were needed, studies were conducted to determine whether bile acid clearances could be utilized to detect occupationally-related hepatic injury. Bile acids are naturally occurring steroids which are synthesized and removed solely by the liver. These substances, in contrast to synthetic anionic dyes such as indocyanine green, have been reported to provide equal if not better, sensitivity in the detection of liver disease. The development of radioimmunoassays for bile acids provided an economical means of determining whether these natural substances could be used as effective screening measures in subclinical liver disease. Studies were conducted to determine the relative effectiveness of bile acid levels vs. ICG clearances in the detection of subclinical liver disease.

Objective

1. To determine the effectiveness of bile acid clearance in the detection of subclinical liver injury

2. To compare bile acids to ICG clearance in identifying chemical and non-chemical liver injury.

Research Results

A subcohort (64) of the worker population who had liver biopsies done for medical reasons and had both ICG clearances and serum bile acids--cholylglycine (CG) and conjugates of cholic acid (CCA)--performed, were studied. These individuals were subdivided on the basis of the histological features of their biopsies into individuals with chemical liver injury (CLI), individuals with non-chemical liver disease (NCLD), and individuals with no histological abnormalities (NBX). The mean plus or minus S.E.M. for ICG, CG and CCA in the CLI, NCLD, NBX, and a non-biopsied normal group are shown in Table 2.

TABLE 2

CORRELATION OF LIVER BIOPSY WITH
BILE ACID LEVELS AND ICG CLEARANCE

	CLI	NCLD	NBX	NON BIOPSIED NORMAL
ICG(%) (0.5 mg/kg)	4.2 ± 0.6	3.2 ± 0.1	3.3 ± 0.2	3.1 ± 0.01
CG (ug/dl)	95.2 ± 28.3	27.3 ± 4.4	34.60± 7.1	14.9 ± 0.9
CCA (ug/dl)	89.7 ± 29.3	25.3 ± 4.5	52.6 ± 26.6	18.7 ± 1.2

Analysis of variance (on log transform data) showed significant differences for ICG clearance for the three biopsied groups and for all four groups. Values for the CLD were significantly different from the ICGs for the normal, but showed no significant difference between the NCLD and the NBX group. Analysis of variance for ICG clearance was not as discriminating for the 3 biopsied groups. ICG clearance gave a better separation (sensitivity) of the normal vs. abnormal at the 95 percentile level for the normal population in the serum bile acids.

This preliminary analysis, therefore, shows that fasting serum bile acids hold promise as a potential detector of early liver dysfunction, particularly chemically-induced, in those individuals who are asymptomatic. This test should be further investigated to determine how this natural substance might be more effectively utilized as a screening technique. Bile acids can be taken orally for clearance study and are not limited to intravenous route as is the case for synthetic anionic dyes such as ICG. Studies in this area continued to be pursued in similar populations.

Relevance to Industry

These clinical study results provide the best scientific basis upon which to recommend effective medical screening tests for the detection of occupationally-related injury to the liver. The results also provide sound scientific reasons for recommending modification of presently required federal testing in environments utilizing potentially hepatotoxic agents.

Although synthetic anionic dye presently provides the best means for detecting early latent liver injury, it is limited by the need to be injected (an invasive procedure). Although reactions to the injection of this dye have been minimal, it does present barriers to voluntary worker compliance. Therefore, development of a screening test which can effectively determine

overall hepatic function, can be given orally, and is a nonsynthetic, normal biological constituent approaches the most ideal test for screening. Bile acid clearance could be the least invasive and therefore the most acceptable method to test asymptomatic individuals undergoing screening. The bile acid studies described above are the basis for recommending its use in future screening. The standardization of these bile acid test techniques, however, requires further careful validation so that specific recommendations regarding their use will provide the most accurate delineation between those with disease and without disease and those with occupationally-related disorders versus naturally-occurring ones.

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PROGRAM C

STUDY OF GLYCOSAMINOGLYCAN CHANGES IN THE DETECTION OF HEPATIC FIBROTIC INJURY IN CHEMICAL EXPOSURE AND HEPATIC CANCER DEVELOPMENT; Investigators - C.E. Kupchella and R. Warick

ANIMAL STUDIES:

C1. The Study of Glycosaminoglycan Changes in Livers of Animals Bearing Metastasizing Hepatomas

C2. Significance of Tissue Glycosaminoglycan Elevations in Hepatic Fibrosis, Necrosis, and Regeneration

HUMAN STUDIES:

C3. Characterization of Glycosaminoglycan Patterns in Humans with Angiosarcoma

C5. Characterization of Glycosaminoglycan Patterns in the Identification of Chemical and Nonchemical Injury of the Liver

C4. Evaluation of Glycosaminoglycan Analysis of Urines of Chemically- Exposed Workers

Background

It is well established that glycosaminoglycans (GAGs) are involved in wound healing and scar formation. Although it is less certain what role they play, it is known that certain GAGs are elevated in malignant tumors including hepatic tumors, and it has been postulated that GAGs may be impor- tant determinants of tumor-cell properties. A number of laboratories, including ours, have established that urinary GAG excretions may serve as markers in the pathogenesis of chemical injury, fibrosis, and cancer. Despite the fact that urinary GAG analyses have long been used clinically to detect and diagnose genetically-determined metabolic disorders of GAG metabolism, a

systematic evaluation of the usefulness of urinary GAG patterns in the detection and diagnosis of acute and/or chronic, necrotic and/or fibrotic liver injury--or cancer--has never been made. Studies were begun to determine the practical utility of tissue and urinary GAG analysis in the detection and diagnosis of chemically-induced liver injury and cancers.

Objective

In Animals:

1. To determine the relationship between GAG patterns in tumor tissue (and urine) in animals with fast versus slow growing and in metastasizing versus non-metastasizing, chemically-induced, transplantable, hepatocellular tumors. (As an initial means of exploring the functional role of GAGs known to be elevated in hepatic cancer).

2. To determine the sequence of GAG changes (in tissue, urine and blood) associated with the onset of experimentally-induced fibrotic injury with special attention to any change coinciding with the transition from reversible to irreversible fibrosis.

3. To determine the degree to which chemically-induced necrosis of the liver results in altered urinary GAG excretion and confirm changes seen in tissue in earlier studies.

In Humans:

4. To compare the relative accuracy of the common methods of evaluating urinary GAGs and find the least expensive and least time-consuming method of urinary GAG analysis able to give good specificity and sensitivity.

5. To repeat in a double-blind clinical trial the evaluation of the ability of urinary GAG analysis to correctly identify active liver disease.

6. To evaluate urinary GAG patterns in groups of alcoholics with active liver injury over a time period.

7. To compare liver tissue and urinary GAG patterns in patients (a) with alcohol-injured livers, (b) hepatic angiosarcoma, (c) primary hepatocellular tumors, (d) livers with metastatic cancer, and (e) chemically-induced hepatitis and cirrhosis.

Research Results

In Animals:

Livers of animals bearing metastasizing hepatoma (5123tc) have 10-fold greater concentrations of a non-sulfated, neutral, uronic acid-positive

material than is found in the livers of animals bearing two others, non-metastasizing hepatomas (4).

Heparan sulfate and hyaluronic acid levels--but not heparin--are 3-4 times higher in experimentally transplanted hepatomas than in normal liver and urinary excretion reflects both the tumor GAG composition and the size of tumors (4, 8).

Hepatic necrosis is accompanied by significant tissue GAG elevations but hepatic regeneration is not (5).

Heparan sulfate (a type of GAG) is elevated in hepatic tissue undergoing experimentally induced fibrosis and heparan sulfate is elevated in the urine of experimental animals (3).

In Humans:

Human hepatic angiosarcoma and fibrotic liver disease are accompanied by elevated tissue GAGs (1).

The GAGs in the angiosarcomatous tumor tissue are different from those in fibrotic tissue adjacent to the tumor (2).

Angiosarcoma and hepatoma patients have characteristic urinary GAG patterns--patterns not found in normal controls (2).

Gross (non-fractionated) urinary GAG determinations give a better indication of liver disease than ultrasound analysis. Exacting urinary GAG analysis (fractionated) is able to differentiated active from inactive liver disease (7). Table 1 illustrates this for chrondrotin sulfate fraction.

TABLE 1

PRELIMINARY EVALUATION OF THE USE OF AN EXCLUSIVELY CHONDROITIN SULFATE EXCRETION PATTERN AS A SCREENING TEST FOR VINYL - CHLORIDE - EXPOSURE - ASSOCIATED LIVER INJURY *

TEST* RESULT	VINYL CHLORIDE EXPOSURE PLUS NON-ANGIOSARCOMA LIVER INJURY	
	+	-
+	7	3
-	2	29
SUM	9	32

SENSITIVITY = 77.7% (95% LIMITS 40.0% - 97.2%)

SPECIFICITY = 90.6% (95% LIMITS 75% - 98%)

*URINE FRACTIONATION IN WHICH THE CHONDROITIN SULFATE FRACTION IS POSITIVE BUT THE HYALURONIC ACID AND HEPARIN FRACTIONS ARE BOTH NEGATIVE.

Relevance to Industry

These studies address the need for useful screening tests for liver injury--urine tests of the type evaluated obviously fit the ideal of being non-invasive, have no associated morbidity/mortality, and do not require "time off" from work to perform.

They could provide useful screening tests for early injury and for differentiating active versus inactive disease.

GAG analysis may help assess methods of therapeutic intervention, i.e., the elucidation of the role of the GAGs in the pathogenesis of fibrotic liver disease and the identification of strategies by which fibrogenesis can be blocked and/or reversed.

Finally, they could provide useful means of identifying individuals at risk of chemical injury--by further characterization of GAG changes, one may be able to identify specifc injury due to chronic alcohol, chemical or other exposure agents in individuals with liver impairment.

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PROGRAM D

HISTOLOGIC AND MORPHOMETRIC ANALYSIS: A MEANS OF ACCESSING HISTOLOGICAL HEPATIC INJURY IN CHEMICAL WORKERS; Investigators - G.H. Barrows, R. Schrodt, and C.H. Tamburro

D1. Morphometric Assessment of Histological Lesions Characteristic of Vinyl Chloride Injury

D2. Computer-Assisted Morphometric Analysis as a Rapid Means of Determining Collagen Content

D3. Development and Assessment of Morphometric Method of Analysis of Collagen Content from Human Liver Biopsies - Relationship to Age

D4. Light Microscopic Assessment of Various Histological Lesions Found in Liver Biopsy Tissue Obtained from Vinyl Chloride Workers

Background

Observations made on liver biopsy specimens obtained from vinyl monomer polymerization workers have identified the association of periportal, subcapsular, and mid-zonal fibrosis, and activated sinusoidal lining cells with vinyl chloride exposure. Many have believed these findings to be early indicators of potentially dangerous chemical exposure since these histological findings are characteristic patterns in the terminal or late stages of disease.

The early stages of increased collagen deposition are often difficult to identify due to a lack of data concerning the normal collagen content and normal variation of collagen in the sinusoidal spaces. Other types of chemical workers' injuries, such as copper smelters' arsenic-induced injury, are also associated with increased collagen formation especially in the perisinusoidal areas (1-3, 6-7). In addition, there had been no systematic analysis of the collagen content of the liver's parenchyma based on age. Only subjective data was available regarding "normal" increases in the quantity of fibrous tissue within the aging liver. Therefore, objective criteria were needed to determine (a) whether the increased collagen deposition in industrial workers could be used as an indicator of developing cancer, and (b) whether this increased collagen could be identified solely on tissue obtained by liver biopsy.

Industrial vinyl monomer exposure, especially vinyl chloride, has been associated with various hepatic histological abnormalities. These have included hyperplasia of both the hepatocytes and sinusoidal cells. Early histological studies, mainly on autopsy material, have shown focal mixed hyperplasia (hyperplasia of the hepatocytes or sinusoidal cells) to be an early histological alteration associated with vinyl chloride exposure (4,7,8). A double-blind histological assessment was conducted on the liver biopsies of some 120 exposed chemical workers to substantiate this observation and determine its potential use in medical surveillance. Assessment of the degree and duration of the vinyl chloride exposure was determined utilizing rank ordered exposure indices as reported by Greenburg and Tamburro (9).

Objectives

1. Develop a clinically usable method for morphometric analysis of collagen content of liver biopsies.

2. Determine whether computer-assisted morphometric analysis could be used as the standard means of determining collagen content from human liver biopsies.

3. Morphometic assessment of normal collagen content and changes associated with increasing age.

4. Review and analyze light microscopic findings of liver tissue obtained from vinyl chloride workers to determine whether specific histological lesions were sufficiently characteristic to be identified and associated with vinyl chloride exposure.

Research Results

(1) Initially, a manual calculator-assisted approach was used for quanitation of trichrome stainable collagen in liver biopsies. Randomly selected areas of the liver biopsy were photographed. These were projected in an 16 x 16 cm area on a Hewlett-Packard 9864A digitizing plate and area calculated using a Hewlett-Packard 9815 calculator. Measurements of collagen content and distribution of the perisinusoidal spaces were made from a randomly selected number of complete grid sections in the various sublobular zones; they were prenumbered from left to right as shown in Figure 1A. The areas were measured in micrographs by superimposing an array of sampling points (Figure 1B) and measuring the number of those points which overlaid specific areas. Two randomly selected grid areas from 3 histological, (periportal, mid-zonal, central) and 4 anatomical locations by 2 methods were studied from 2 to 3 individuals in each bracket.

Portal to central distance was quantitated and divided into 3 sections: pericentral, midzonal, and periportal. A trained observer then manually

outlined all areas staining as collagen and the total sinusoidal area (Figure 2). This was repeated for a total of 3 areas in each biopsy sample. These randomly selected areas provided sufficient sampling to obtain a reproducible estimate of collagen content of various areas of liver lobule and were highly reproducible (Table 1).

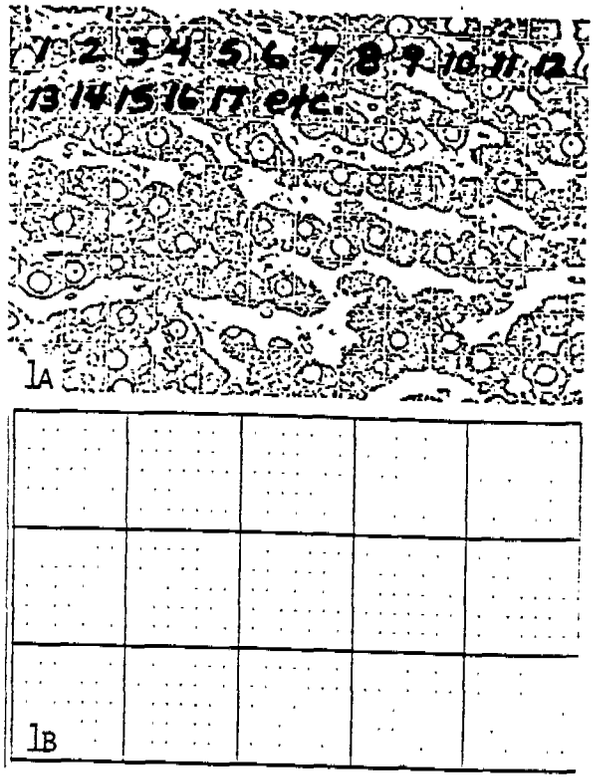


FIGURE 1

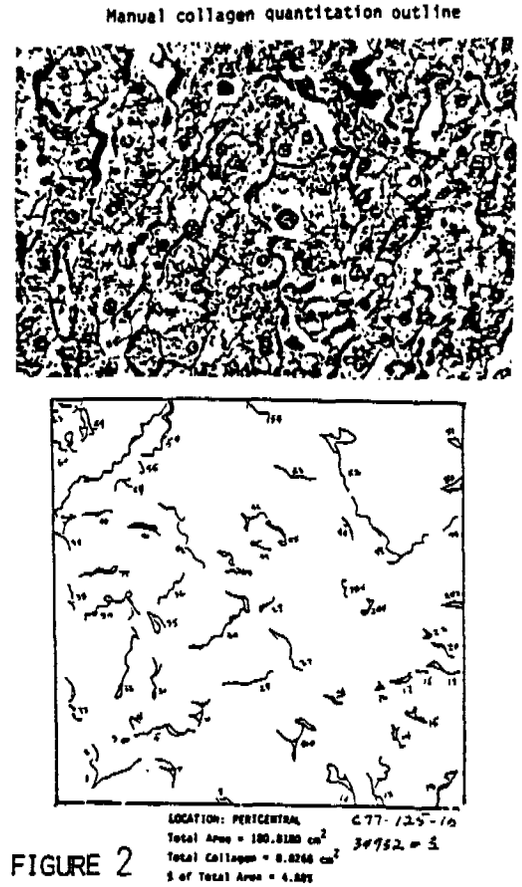


FIGURE 2

TABLE 1

REPRODUCIBILITY

Area Percent	Digitizer		Square Counting
	Inter assay	Intra Assay	
2.03	0.40	0.018	0.27
4.30	0.19	0.052	0.35
6.10	0.55	0.054	1.10
14.70	1.27	0.6075	2.95

(2) Later developments for computer quantitation promise to facilitate the execution of morphometric collagen determination. In this approach a video microscopic image is digitized to 4 bit precision, and held in digital video storage (Morphometrix-156 Image Analyzer). This digital image (Figure 3A, 3B) contains all density information on a 256 x 512 matrix. The amount of staining of a particular density can be rapidly quantitated by counting the percentage of a particular density which occupies an image. This approach allows rapid quantitation of the total biopsy and enables study of a large number of samples. A second advantage is the adaptability of this technique for 3-dimensional reconstruction and quantitation of liver fibrosis volume. Figure 3 illustrates a computer printout of a histological area and a drawing of the area morphometrically analyzed.

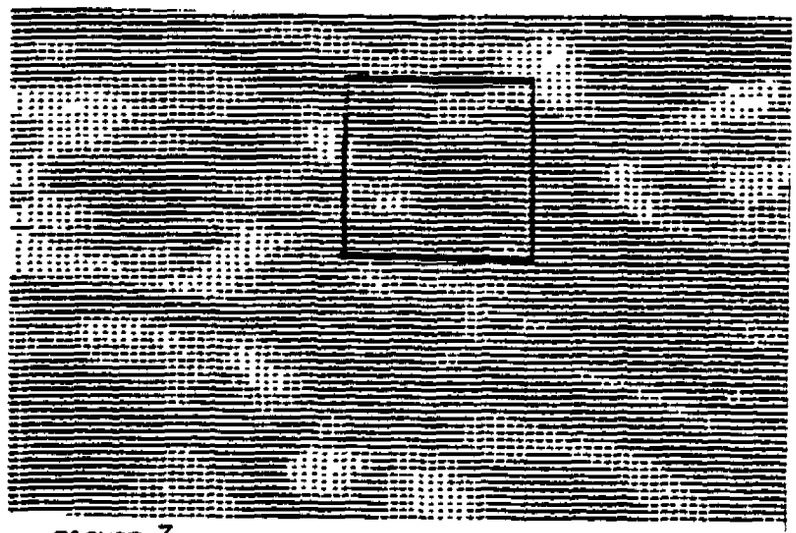


FIGURE 3A: Density printout of Figure 2, with 10:1 reduction (area in square corresponds to digital printout 3B).

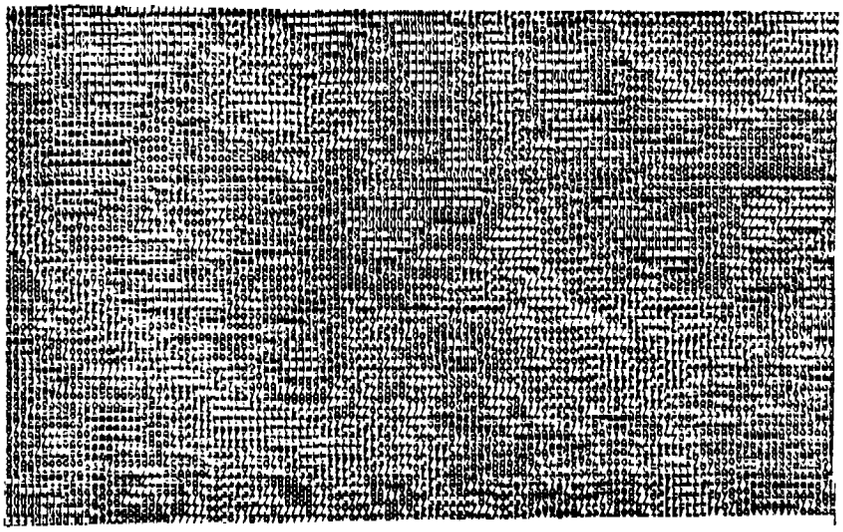


FIGURE 3B Digital densities of reticulin stained liver biopsy.

(3) Histological tissue was obtained for the study of liver collagen content at various ages from individuals dying of sudden death without historical, clinical or autopsy evidence of hepatobiliary disease, chronic congestive heart failure, excessive alcohol consumption, exposure to hepatoxins or infectious agents known to produce long term hepatic injury, drug abuse, or prolonged medical use of drugs known to be hepatoxic or potentially hepatoxic. The groups were divided into four age brackets: 16-30, 31-45, 46-60, 60 and older. Autopsy tissue was obtained from areas of the liver most frequently obtained in living patients by (A) percutaneous needle, (R, L) open wedge, and (D) transjugular biopsy. Needle and wedge samples were obtained from 4 areas of the liver as illustrated in Figure 4.

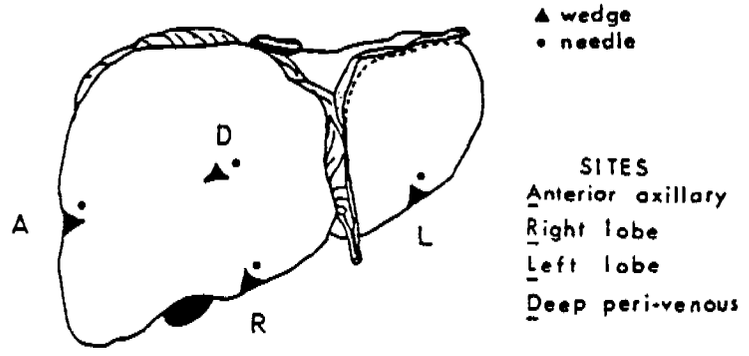


FIGURE 4

Two hundred and fifty three light micrographs derived from 30 tissue blocks from 8 normal individuals were studied. A Hewlett Packard 9864-A digitizer microcomputer was used to quantitate areas of trichrome stainable collagen. Collagen estimates varied from .2 to 6.1% (mean = 1.25) with considerable variation even in different biopsies from the same patient. Subcapsular biopsies have more collagen than deep biopsies; however, deep biopsies have more variation in collagen content. Collagen content appears to increase with age and more in midzonal regions than in portal areas (Table 2).

TABLE 2

PERCENT COLLAGEN RATIO IN THE VARIOUS LOBULE ZONES

AGE	PERICENTRAL / MIDZONAL	PERIPORTAL / MIDZONAL	PERICENTRAL / PERIPORTAL
15	11.8: 1	8.6: 1	1.4: 1
23	2.8: 1	1: 1	2.4: 1
40	2.8: 1	1: 1	2.3: 1
50	1.8: 1	1: 1	1.9: 1
51	1: 1	1: 1	1: 1

This increase appears to become detectable in the 4th and 5th decades (Figure 5).

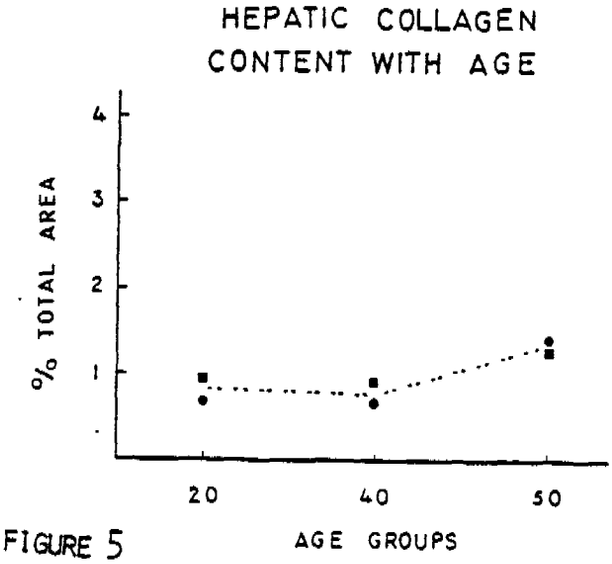


FIGURE 5

Differences in central, mid-zonal and portal collagen vary from 0.49 - 0.63 percent in the youngest age group and from 4 - 5.35 percent in the older age group. This histological data is consistent with the ICG clearances in normal adults which show a progressive decrease with age. The decreased functional capacity of normal aging liver may be due to an increased collagen deposition.

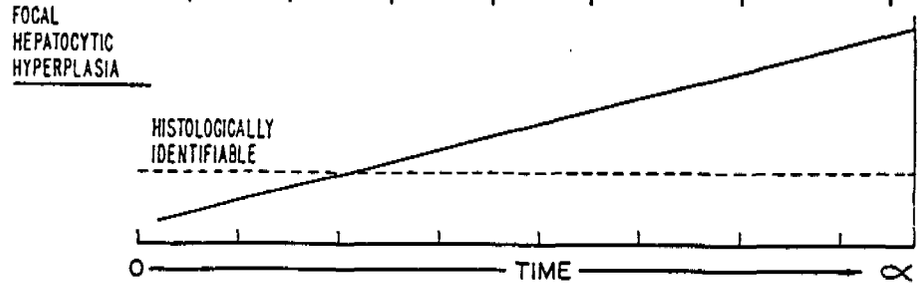
(4) Light microscopic assessment of liver tissue obtained from vinyl chloride workers with varying histological lesions was done. In order to substantiate the ability to identify early histological alterations indicative of chemical exposure, 93 liver biopsies from 78 individuals were investigated duplicatively in a double blind manner. These histological lesions included focal hepatocellular hyperplasia (Figure 6), focal mixed hyperplasia, sinusoidal dilitation (Figure 7), focal areas of increased reticulum (Figure 8), and focal subcapsular fibrosis (Figure 9). The progressive development of vinyl chloride liver lesions are illustrated in Table 3.

Thirty-five of these individuals were exposed chemical workers with hepatic screening test abnormalities, and 13 were exposed workers without hepatic screening test abnormalities who had had liver biopsies for non-liver related medical reasons. A group of 30 individuals who were not chemical workers, who had liver biopsies for nonhepatic-related illness during the same period of time at the same hospital, were compared. Twenty-three (48%) of the exposed workers had hepatic lesions consistent with exposure, 17 (35%) had only focal hepatocellular hyperplasia, and 6 (13%) had focal mixed hyperplasia or more advanced lesions.

TABLE 3

PROBABLE PHASE DEVELOPMENT OF VINYL CHLORIDE PRE-CANCEROUS LESIONS

CLINICAL FINDINGS	NONE	BIOCHEMICAL ABNORMAL	PORTAL HYPER-TENSION	SPLENO-MEGALY	HEPATIC SCAN ABNORMAL	HEPATIS PELIOSIS	CANCER
HISTOLOGICAL FINDING	NORMAL	FOCAL HEPATOCYTIC HYPERPLASIA	PERI-SINUSOIDAL FIBROSIS	FOCAL MIXED HYPERPLASIA	PORTAL AND CAPSULAR FIBROSIS	SINUSOIDAL CELL DYSPLASIA	ANGIO-SARCOMA



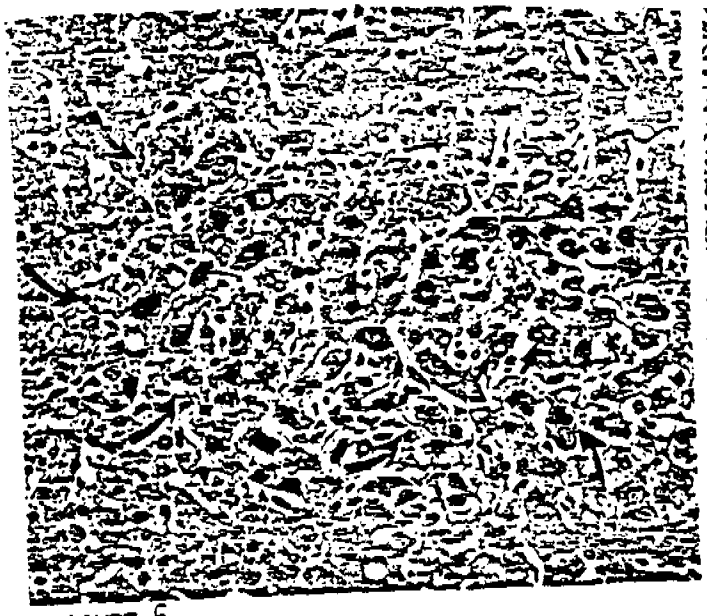


FIGURE 6

FOCAL HYPERPLASIA OF HEPATOCYTES WITH GREAT VARIATIONS IN THEIR SIZE AS WELL AS THEIR NUCLEI. THE LATTER ARE FREQUENTLY DOUBLE, POLYCHROMATOPHILIC OR VACUOLATED. H&E, 150x.

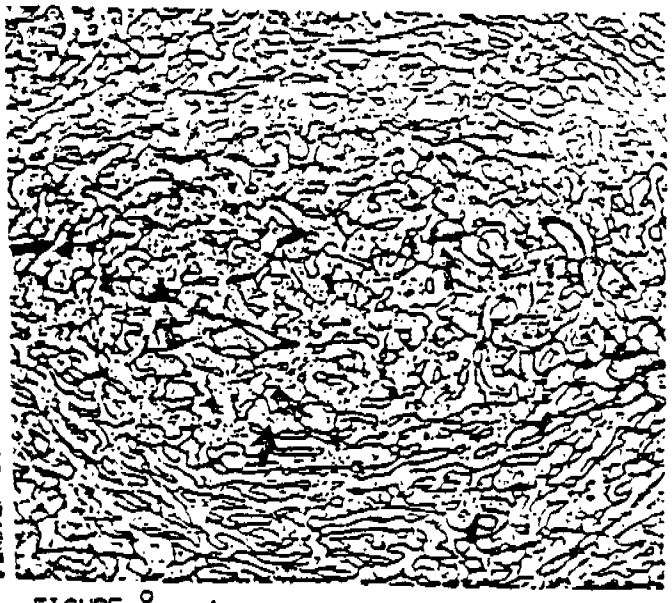


FIGURE 8

FOCAL INCREASE OF RETICULIN FRAMEWORK. SILVER IMPREGNATION, 100x.

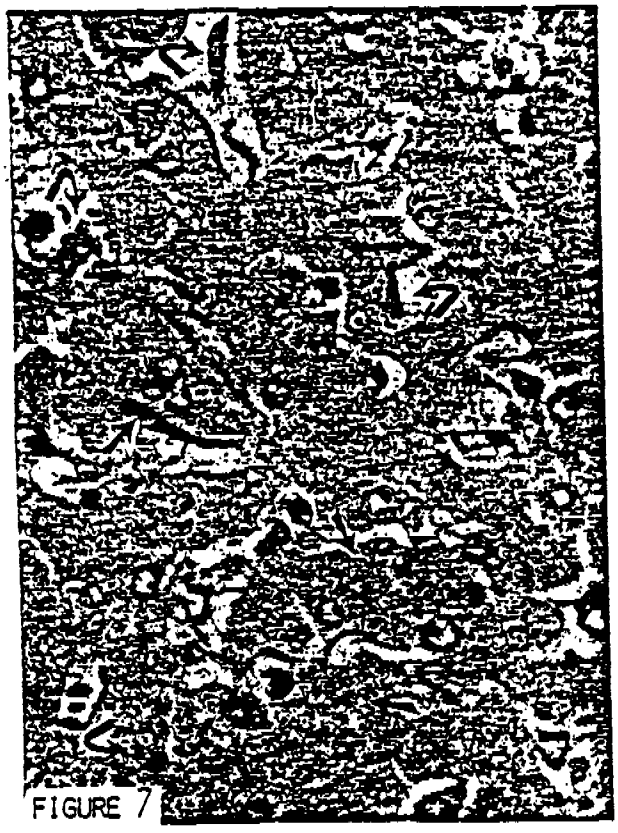


FIGURE 7

HYPERPLASIA OF SINUSOIDAL CELL (CURVED ARROWS) AND HEPATOCYTES (STRAIGHT ARROWS). (H&E, 100x)

MIXED HYPERPLASIA

FIGURE 9

FOCAL CAPSULAR AND SUBCAPSULAR FIBROSIS. ANILINE BLUE, 60x.

In contrast only 5 of the comparison group had similar findings. Four (13%) had focal hepatocellular hyperplasia, and 1 (3%) had focal mixed hyperplasia and sinusoidal dilitation. On subsequent biopsy this individual was found to have angiosarcoma and a history of using hair spray containing vinyl chloride as a propellent.

Ten individuals had 28 biopsies reviewed double blindly, and 10 individuals had 23 readings of the same biopsy; 21 of 23 (91%) duplicate readings and 27 of 28 (96%) multiple biopsy readings in the same individuals were identical. Only 18% of the individuals (3 of 17) had either duplicate and/or multiple biopsy readings which disagreed with their prior biopsy assessment. Focal hepatocytic hyperplasia, in addition to the previously described mixed hyperplasia, appeared to be the earliest identifiable change consistent with chemical exposure. These lesions can be consistently identified and are useful in screening chemical workers for evidence of chemical exposure.

Chemical exposure histories to 20 different occupational chemicals were analyzed with regard to the histological findings in the liver biopsy. Portal tract fibrosis, disruption of the limiting plate, and changes in the limiting plate of the portal area had a significant correlation in individuals identified as having histological features of chemical liver injury in contrast to those who had nonchemical liver disease. A larger percentage of individuals with histological evidence of chemical injury had higher levels of exposure to vinyl chloride (Figure 10). Changes in the sinusoidal areas also demonstrated the highest correlation with vinyl chloride's cumulative exposure rank months, but not with the cumulative exposure rank months of acrylonitrile, a comparison chemical.

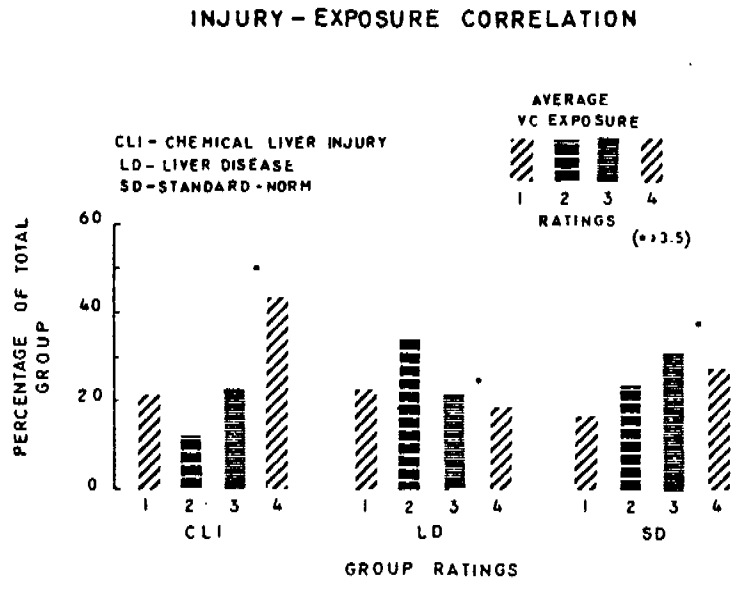


FIGURE 10

Relevance to Industry

These studies demonstrate some highly important and critical information regarding documentation of chemical exposure-induced liver injury:

(1) Rapid methods of quantitatively assessing total collagen content in human liver tissue have been applied. These can provide both retrospective as well as prospective analysis.

(2) Morphometric analysis of collagen can be performed for clinical purposes from human liver biopsies. This can be automated by using a computer for large volume work.

(3) The identification of increased liver collagen content with age demonstrates the need for better standards of "normality" before one can properly determine whether increased liver collagen is a reflection of chronic injury that may be work-related.

(4) There are histologically identifiable lesions that are consistent with chemical injury; these lesions can be separated from those histological findings of nonchemical liver injury. These characteristic chemical liver injury lesions are correlated with vinyl monomers, most significantly vinyl chloride. Therefore, at the present state of knowledge, one can reasonably determine the presence or absence of significant chemical exposure from the examination of human liver tissue. The present studies have not demonstrated any spontaneous progression in the earliest lesions, i.e., focal hepatocellular hyperplasia when found alone. . The present opinion is that lesion may simply reflect cellular adaptation to chronic exposure while mixed hyperplasia may indicate a higher risk of future cancer development.

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PROGRAM E

STUDIES OF VINYL MONOMER CHEMICALS AND THEIR METABOLITES USING CHEMICAL STRUCTURE AND SYNTHESIS IN THE DETERMINATION OF TOXICITY OF THESE AGENTS; Investigator - J. L. Wong

E1. Synthesis, Purification, and Utilization of Vinyl Chloride Metabolites in Mutagenicity and Carcinogenicity Studies

E2. Detoxification Studies of Vinyl Chloride and Its Metabolites

E3. Vinyl Chloride Metabolite Detection--Chloroacetic Acid

Background

The mutagenicity of vinyl chloride and its potential metabolites was studied in detail by a combination of chemical synthetic and microbiological assay techniques. We prepared and characterized the potential putative metabolites (chlorooxirane and four forms of chloroacetaldehyde), and Dr. Uldis Streips of Microbiology conducted the testing of these compounds in the Bacillus and Salmonella systems.

Since all literature methods for the preparation of chlorooxirane result in mixtures with contaminants such as chlorine, hydrogen chloride, ethylene oxide, polymers, etc., it was necessary to develop a method to prepare pure chlorooxirane.

In order to elucidate the intermediary metabolism of vinyl chloride, i.e., the metabolic activation and inactivation steps, to determine the significant end products of vinyl chloride in body tissues and fluids, the fate of chlorooxirane, the immediate P450 product of vinyl chloride, was studied using synthetic chlorooxirane to react with non-protein sulfhydryls.

Another prominent reactive property of chlorooxirane is its facile rearrangement to chloroacetaldehyde (CAA). In dimethylformamide at 25°C, it has a half-life of ~0.5 min in rearranging to CAA. Therefore, the mechanism of deactivation of CAA by cysteine was studied. The plausible cyclic product--3L-carboxy-2,3-dihydro-1,4-thiazine--may be the precursor of the urinary metabolites S-hydroxyethylcysteine and S-carboxymethylcysteine.

As a putative metabolite, CAA is well-known to react with adenine and cytosine nucleotides. We will demonstrate that CAA can also react with guanine derivatives at body temperature and physiological pH to yield covalent products. These products will alter the N-H hydrogen-bonding sites in the nucleic acid bonds, hence their formation in a biological host may be consequential in inducing the onset of neoplasia.

Since previous animal studies have not demonstrated chloroacetic acid in the urines of animals at low exposure levels but have been reported in humans at higher exposure levels, we intend to develop analytical methodologies which will help to verify this observation. Such studies may lead to a method of warning of environmental exposure to humans. The first target metabolite for analysis was chloroacetic acid.

Objective

1. To study the chemistry of vinyl chloride toxicity by identifying the intermediates of vinyl chloride metabolism and their reactions with cytoplasmic chemicals.

2. To determine the putative actions of the primary metabolites with regard to:

   a) the chemical reactions of metabolites, chlorooxirane (COR) and chloroacetaldehyde (CAA) with sulfhydryls (detoxification).

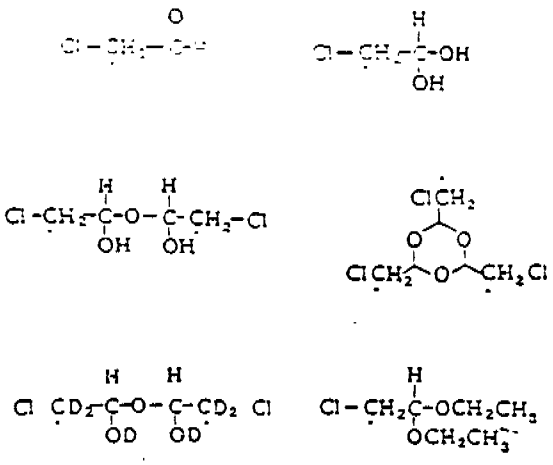
   b) reactions on nucleic acid constituents (mutagenesis and carcinogenesis).

3. To develop analytical methods to detect a target metabolite--chloroacetic acid.

E1. Synthesis, Purification, and Utilization of Vinyl Chloride Metabolites in Mutagenicity and Carcinogenicity Studies

The detection study of the putative action of vinyl chloride is based on the hypothesis that such action comes from the modification of the nucleic acid materials by the primary metabolites COR and CAA. Although CAA is long known to react with nucleic acid bases such as cytosine and adenine to form the etheno derivatives, little is known about the reaction of CAA on the most reactive base, guanine. Our study of CAA with guanine base using analytical tools such as HPLC, GC-MS and FT-NMR have shown that the guanosine reaction is enormously complicated.

The hetero-bifunctional alkylating structures of 2-chloroacetaldehyde are shown below.



Studies of their reactivities have necessitated:

a. Synthesis of etheno-modified components of RNA and DNA— angular-etheno-guanine, linear-etheno-guanine, linear-etheno-guanosine, linear-etheno-deoxy-guanosine, etheno-adenine, etheno-adenosine, etheno-deoxy-adenosine, etheno-cytosine, etheno-cytidine, and etheno-deoxy-cytidine.

b. Synthesis of acidic hydrolytic products of etheno-modified bases— 2-aminobiimidazole, 2-formamidobiimdazole, E1-D-angular-etheno-guanine, and E1-D-linear-etheno-guanine.

c. Synthesis of alkaline decomposition products of etheno-modified nucleosides—2-amino-biimidazole nucleoside and 2-formamido-biimidazole nucleoside.

d. Synthesis of the deuterated analogues for detailed structural study—8-D-etheno-adenine, E1-D-etheno-cytosine, E1-D-angular-etheno-guanine, E1-D- etheno-adenine, and E1-D-linear-etheno-guanine.

The kinetics of guanosine with CAA at wide range pH value have been studied with careful pH control. The pK vs. pH plot showed that there are two plateaus, pH1 and pH8, and there is minimum at pH 4.5. From the product profiles, two different mechanisms can be suggested (Figure 1).

The putative action of vinyl chloride in respect to the physicochemical properties of the etheno derivatives of cytidine, adenosine, and guanosine is further studied. The ^{13}C-FTNMR spectroscopic properties are particularly revealing. By means of deuteration and proton undercoupling technique in acquiring the carbon-13 spectra, the various carbon chemical shifts are assigned and the ^{13}C-^{1}H coupling constants determined. From the latter data, the conformational preferences of the sugar moiety in the modified nucleosides are deduced as follows: (1) anti conformation for ethenocytidine

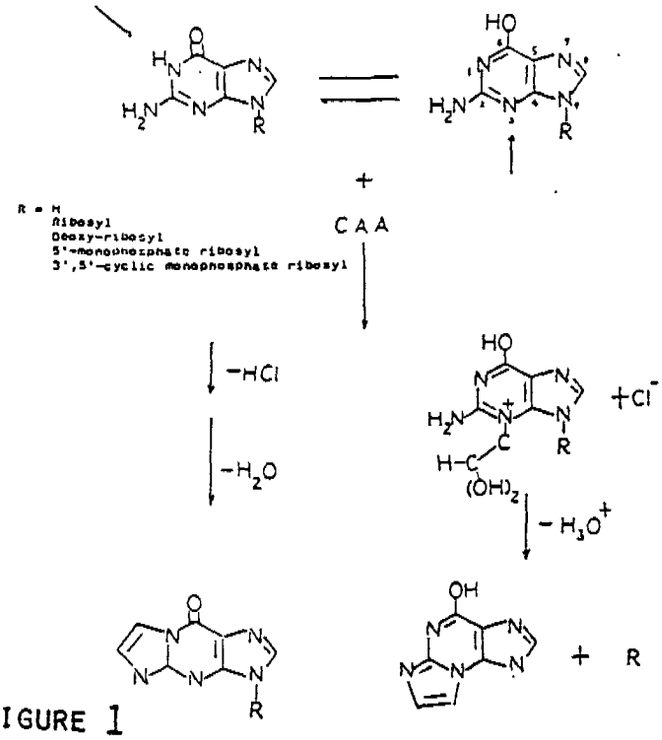


FIGURE 1

and (2) syn for etheno-adenosine. Since anti conformation of nucleosides is necessary for the formation and stability of the helix, the influence of the etheno bridge on the adenine nucleus in altering the ribosyl group to syn will impose considerable stress on the DNA chain which might result in biological damages.

E2. Detoxification Studies of Vinyl Chloride and Its Metabolites

A previous attempt by Gothe et al. to trap the putative metabolites COR and CAA in vitro from vinyl chloride with 3,4-dichlorobenzenethiol has led to the identification of the product as 3,4-dichlorophenylthioacetaldehyde. It was interpreted to be indicative of the formation of either COR or CAA. We have defined this experiment further. Thus, chlorooxirane in organic medium reacts slowly with 3,4-dichlorobenzenethiol to form 2-(3,4-dichlorophenylthio) acetaldehyde as 80 percent of the products. This reaction at room temperature takes about 7 days. At 60°C the reaction is completed in 21 hours giving the same products. (In both cases some disulfide Cl_2Ph-S-S-Ph-Cl_2 and other unidentified polymeric materials are observed.) The identity of the product was established by comparison of gas chromatography retention time with an authentic example, by PMR and by mass spectrum. In aqueous acetone (2:1) or aqueous acetonitrile (3:1), the reaction is much faster. As pH = 4, 55 percent conversion is observed in 15 min. which is the lifetime of COR under

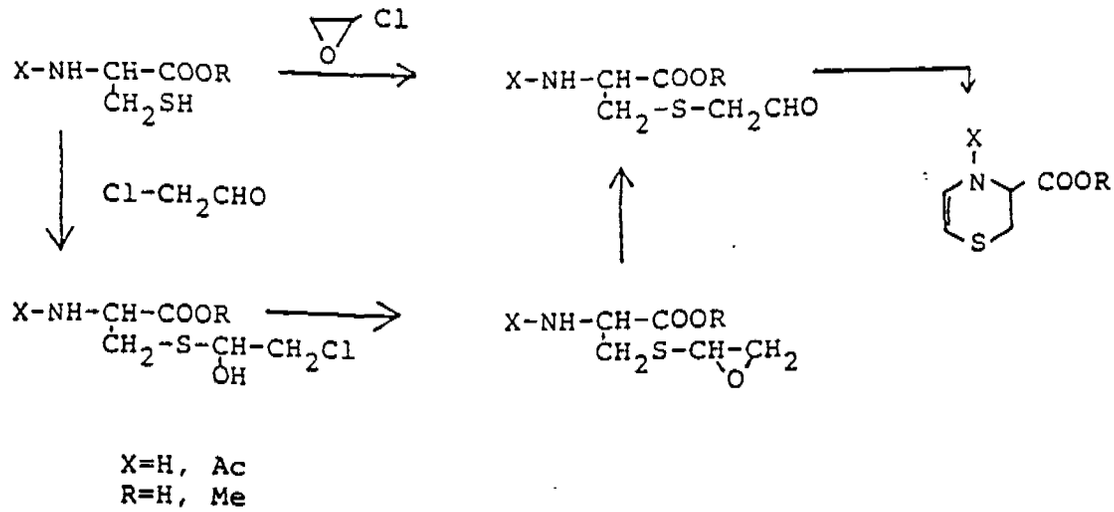
these conditions, and 80 percent conversion at pH = 7. The sole product identified from these reactions by high pressure liquid chromatography is the aldehyde indicated. On the other hand, chloroacetaldehyde in $CHCl_3$ gives another addition product, which was identified by PMR and IR and its facile reversion to the starting materials as $3,4\text{-}Cl_2Ph\text{-}S\text{-}CH(OH)CH_2Cl$. In aqueous acetone or aqueous acetonitrile, CAA did not react with the thiol within 1/2 hour, indicating its lower reactivity to the aromatic SH group compared to COR. These results, combined with the in vitro experiment by Gothe, have confirmed that chlorooxirane is an obligatory intermediate in the metabolism of vinyl chloride.

The detoxification of chlorooxirane was also studied in aqueous media at different pH's. N-Acetylcysteine was used as a typical cellular sulfydryl compound involved in detoxification. In aqueous solutions at pH4 and 7, their reaction was extremely fast at room temperature, yielding N-acetylcysteine-S-acetaldehyde. It was identified by ^1H and ^{13}C NMR and characterized as the 2,4-dinitrophenylhydrazone derivative. This aldehyde is probably the precursor of the urinary metabolites, S-2-hydroxyethylcysteine and thiodiglycolic acid.

The reaction of chloroacetaldehyde with N-acetylcysteine under controlled pH conditions in aqueous medium at 0°C produced an intermediate compound. Upon warming up the reaction mixture to room temperature, our previously identified thiazene was isolated as the final product. In order to elucidate the stepwise formation of the thiazene, N-acetylcysteine methyl ester was allowed to react with chloroacetaldehyde in chloroform at 0°C. The initial product, plausibly the hemithioacetal $H_3CO\text{-}CO\text{-}CH\text{-}(NHCOCH_3)CH_2S\text{-}CHOH\text{-}CH_2Cl$, eliminated HCl upon neutralization with aqueous sodium hydroxide to produce the corresponding epoxide $H_3COCOCH(NHCOCH_3)CH_2S\text{-}CHO\text{-}CH_2$. This structural assignment is supported by its PMR spectrum. Furthermore, when this epoxide was extracted into chloroform, it rearranged to the S-acetaldehyde $H_3COCOCH\text{-}(NHCOCH_3)CH_2\text{-}S\text{-}CH_2CHO$, identified by its pmr spectrum and by comparison with that formed from the reaction of N-acetylcysteine with chlorooxirane.

Even though chlorooxirane and chloroacetaldehyde eventually give the same final product with N-acetylcysteine, the rate of reaction and the intermediates in the two reactions are different. The chlorooxirane conjugates instantaneously with the sulfhydryl compound, while chloroacetaldehyde takes about 2 1/2 hours for a comparable reaction.

The comparative study of the reaction of chlorooxirane and chloroacetaldehyde with sulfhydryl compounds can now be summarized in the following reaction pathways with cysteines (unmodified, N-acetyl, and N-acetyl-methyl ester):



Although both routes are converged to yield the cysteine S-acetaldehyde conjugate, their reaction rates are vastly different. In general, chlorooxirane completes its reaction within an hour while chloroacetaldehyde requires overnight. These chemical observations can be applied in (1) specific assays for the two putative metabolites in cellular studies of vinyl chloride, and (2) detoxification mechanism of cells exposed to the vinyl carcinogen.

E3. Vinyl Chloride Metabolite Detection - Chloroacetic Acid

The methodology includes gas chromatography and mass spectrometry. Gas chromatography results are as follows. Solid supports containing Carbowax and FFAP liquid phases have been used for direct analysis of carboxylic acids. Accordingly, 20 percent FFAP on chromosorb W passes chloroacetic acid at 200° with modest tailing of the peak. However, with this simple column, sensitivity is limited to concentration ranges of 0.2 - 2 mg/ml H_2O (200 - 2,000 ppm). Application of the improvements reported by Ackman (use of porous polymer solid support and use of formic acid in helium carrier gas) is expected to improve sensitivity by 100 fold.

Identification of chloroacetic acid by mass spectrometry was carried out. Chloroacetic acid can be identified by mass spectroscopy of water solutions as dilute as 0.02M (2mg/ml). The 2 doublets of peaks at m/e 49, 51 ($^{35}ClCH_2^+$ and $^{37}ClCH_2^+$) and 50,52 ($^{35}ClCH_3^{+\cdot}$ and $^{37}ClCH_3^{+\cdot}$) are distinctive because relative intensities within each doublet reflect the 76:24 isotopic ratio of $^{35}Cl:^{37}Cl$. Thus, the GC-MS combination technique should be most useful in the detection of the above.

Relevance of Industry

Broadly speaking, this is a study for early detection and prevention of industrial cancers. Our chemical methodologies (synthesis, structure, and analysis), aplied as an integral part of the multidisciplinary approach, contribute to elucidate specific molecular events in the effects of vinyl monomers on industrial workers. This information will form a rational basis for safer use of chemicals and design of preventive measures. Our molecular studies also provide the opportunity to develop useful marker(s) in the form of metabolites in the pathogenesis of chemical injury.

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PROGRAM F

ASSESSMENT OF ASSAYS FOR THE CARCINOGENIC POTENTIAL OF INDUSTRIAL CHEMICALS USING PROKARYTOIC AND EUKARYOTIC SYSTEMS. Investigators; U.N. Streips and G. Sonnenfeld

F1. Bacterial Assays for Testing Carcinogenicity and Mutagenicity of Industrial Chemicals.

Background

Our laboratory had instituted Salmonella reversion, Bacillus subtilis repair, and Bacillus subtilis forward mutations assays for screening the mutagenic potential of chemicals (1-3). For that reason we became an integral part, first, for B. F. Goodrich, then for the Manufacturing Chemists Association sponsored projects on industrial chemical risk assessment.

Objective

1. To screen a series of industrial and environmental - health-related chemicals for mutagenic potential.

2. To initiate with Dr. G. Sonnenfeld a rapid mammalian assay to better predict human-related bacterial chemical activity and carcinogenic potential.

Research Results

In our previous progress reports we have documented the work which delineated the involvement of chloroacetaldehyde and chlorooxirane as the probable active components of vinyl chloride-mediated carcinogenesis. In addition, we screened a multitude of other chemicals generated by Dr. John Wong (our collaborator in this study) or by other investigators in the university or industry (1-3). This is documented in Table 1. Recently, in collaboration with Dr. Gerald Sonnenfeld, a new, exciting, potential screen for carcinogens was developed (4,5). This involved the inhibition of interferon induction and was specific for several carcinogens (including chloroacetaldehyde) but not for non-carcinogenic analogues (including some mutagens). It should be illustrative of some of the molecular mechanisms of carcinogenesis attack on susceptible cells. We continue to develop this system even though our support from CMA has ended (6-8).

A screening laboratory (using microbial assays) for institutional service purposes is being maintained. We have proposed to the state of Kentucky that a regional chemical testing laboratory be funded to serve the industry and university needs in this part of the Commonwealth. That proposal is still pending.

TABLE 1

SUMMARY OF PERTINENT SUBSTANCES TESTED FOR MUTAGENICITY

		Salmonella	Subtilis
1.	Meta-chlorobenzoyl-cyclooctanecarbonyl peroxide	±	NR
2.	Benzoyl peroxide	NR	NR
3.	N-acetoxy-N-phenylacetamide	NR	NR
4.	N-cyclooctanecarboxyloxy-N-phenylacetamide	NR	NR
5.	bis-cyclooctane carboxyl peroxide	NR	NR
6.	Aflatoxin	+++	++
7.	Chloroethanol	NR	NR
8.	Benzo(a)pyrene	++	+
9.	4-nitro quinoline-1-oxide	+++	++
10.	Chloroacetaldehyde	+++	++
11.	Epichlorohydrin	++	+
12.	Chloroxirane	+++	++
13.	Butane diepoxide	++	+
14.	Styrene oxide	++	+
15.	3,4 epoxide butene	±	NR
16.	bis(Beta-chloroethyl)phenyl phosphate	NR	NR
17.	Beta chloroethyl phosphate (bis cyclohexylamine salt)	NR	NR
18.	Lung aspirates from smokers with cancer	±	NR
19.	Lung aspirates from smokers without cancer	±	NR

NR - no reaction + - mutagenic +++ - extremely mutagenic

± - borderline mutagenicity ++ - strongly mutagenic

To further elucidate molecular mechanisms of carcinogenesis, in collaboration with John Wong, we inititated a study of DNA-interaction with carcinogens, and with Ron Doyle, control of cell division processes in bacteria. Progress in this area has allowed us to secure a grant from the National Science Foundation to carry on with the work. Soon, we will be able to describe the cellular controls which maintain proper cell division in bacteria, then relate these findings to mammalian cells. At that time, we will be able to examine carcinogen attack on these processes and determine which events lead to loss of cell division control and neoplastic growth.

Support from the CMA has been instrumental in generating and maintaining research activity in our laboratory relating to carcinogenesis and cell cycle events. We deeply appreciate this support.

Relevance to Industry

With the tremendous number of chemical formulations generated yearly, a battery of rapid screens are crucial for examining the environmental and health-related impact of these chemicals. A regional laboratory can do such screening effectively and economically as shown by the results previously described. In addition we have demonstrated that such research also leads to innovation in terms of new assays (the interferon assay), and related research (the studies on cell division). Industrial support also has led to other granting. Valuable mutagenesis information has been provided to industry regarding crucial chemicals. In turn, industry has provided us with the opportunity to expand and solidify our research efforts. The interaction has been optimal.

F2. Preliminary Studies on the Use of Inhibition of Interferon Induction as an Indicator of Mutagenicity and Carcinogenicity of Chemicals

Background

Interferon, which was originally described as an antiviral agent, has since been shown to have several other activities, including an antitumor activity (9). Clinical trials are now in progress to determine the efficacy of interferon as an anti-cancer agent (10).

We became interested in the interactions of carcinogens, or cancer-causing substances, and interferon. Early studies by DeMaeyer and others suggested that there was indeed an interaction between carcinogens and interferon induction (11-13). Pretreatment of rat embryo fibroblasts with the carcinogens triethyleneamine, 4-nitroquinoline-N-oxide, benzo-(a)-pyrene, and 3-methylcholanthrene all inhibited the induction of alpha-beta interferon by viruses, without affecting the gross viability of the cultures or the replication of the viruses (11-13). Several poorly or noncarcinogenic analogs, such as benzo-(e)-pyrene, had no effect on the production of interferon (11-13). The effect was observed after only one day of incubation of very small amounts of carcinogen with the tissue cultures. In addition, Hahon and co- workers have shown that incubation of cell cultures with various forms of aflatoxin also inhibited the induction of interferon in the cultures (14). In this case, the degree of inhibition correlated with the in vivo carcinogenic potential of the aflatoxin form (14). Asbestos fibers and coal dust have also been reported to inhibit interferon induction in tissue culture (15,16). In vivo, injection of methylcholanthrene into mice inhibited the induction of alpha-beta interferon (17). These results created an interest in our laboratory to further study this phenomenon.

Objective

1. To repeat the early studies to determine if carcinogen pretreatment inhibited interferon induction.

2. To begin studies to determine if effects on interferon induction could be used as a reliable indicator of carcinogenic potential of chemicals.

3. To study the mechanisms of the observed effects.

Research Results

Our first step was to establish the system in a mouse culture model, since this was more appropriate to current interferon technology. Mouse embryo tissue cultures were prepared, treated with carcinogen or analogue for 24 hr., washed with fetal bovine serum-containing medium and with serum-free medium to help remove carcinogen, challenged with an interferon inducer (either Newcastle disease virus or poly I:C), and then assayed for antiviral activity. Earlier data was able to be duplicated and those findings extended. Several known carcinogens including 7,12-dimethylbenz-(a)-anthracene, benzo-(a)-pyrene, 2-aminofluroene, aflatoxin-B_1 and the #4 fraction of tobacco smoke condensate all inhibited interferon induction (5). Styrene oxide, an important industrial chemical which was positive in the Ames Salmonella assay but negative in all Bacillus assays done to date (11), also inhibited interferon induction. Ethyl methanesulfonate (EMS) and methyl methanesulfonate, differ with respect to their carcinogenic potential as determined by tumor induction (18,19). MMS is a mutagen and highly carcinogenic, while EMS is a potent mutagen but rarely carcinogenic. In our assay, MMS strongly inhibited alpha/beta interferon induction, while EMS had no effect. Additional studies in our laboratory extended these findings to the potent carcinogen chloroacetaldehyde and its noncarcinogenic analogues chloroethanol and chloroacetic acid (4). Only chloroacetaldehyde inhibited interferon induction.

An additional finding of interest was the apparent requirement for activation of carcinogens before they could inhibit interferon induction. In a preliminary study, we were able to show that addition of reduced glutathione to our cultures blocked the inhibition of interferon induction by benzo-(a)-pyrene (4). Reduced glutathione can block the active metabolites of carcinogens (20), which suggested that these active metabolites were required for the effects of benzo-(a)-pyrene on interferon induction.

Relevance to Industry

We believe these studies to be highly relevant to the chemical industry because: 1) the studies yielded information on the effects of toxic chemicals on the interferon system, an important body defense against cancer, and 2) the studies may eventually lead to a new assay system for discriminating between carcinogenic and noncarcinogenic chemicals.

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PROGRAM G

THE STUDY OF TISSUE ANTIGENS AND ANTIBODIES IN THE DETECTION OF VINYL CHLORIDE INJURY; Enrique Espinosa, M.D.

G1. The Study of Tissue Antigens from Liver Tumors, Angiosarcoma, and Hepatomas.

G2. Circulating Antigens and Autoantibodies in Vinyl Chloride-Associated Liver Disease

Background

In hepatic fibrosis and angiosarcoma associated with vinyl chloride exposure of industrial workers, manifestations of the disease could not be detected in most cases until the process was far advanced (1). Normal values of liver function tests were reported in a case with significant vinyl chloride hepatic fibrosis (2), and only a small percentage of workers of a plant unit where 7 cases of liver angiosarcoma were diagnosed had abnormal blood screening tests (3). Thus, conventional liver function tests do not appear to be sensitive indicators of vinyl chloride liver disease. Development of more sensitive and specific methods for detecting the disease in early stages would be of great importance. An approach to this may be provided by antigenic studies in view of the tissue antigenic modifications which are known to occur in neoplasia. For example, in human carcinoma, loss of antigens have been reported in squamous cell carcinoma (4,5) and in ovarian carcinoma (6,7), loss of the ABH blood group isoantigens in some solid tumors (8,9), and of HL-A isoantigen in lymphoma (10). Also, tumor-specific transplantation antigens have been demonstrated in a number of experimentally induced tumors (11-14) as well as tumor-associated antigen in spontaneous tumors in man (e.g. 15-18). In such an antigenic study of liver angiosarcoma the question arises whether the fibrotic and angiosarcomatous livers contain antigens that are quantitatively or qualitatively different from those present in normal tissue and whether such changes could stimulate an immunologic response. If such hypothetical changes are demonstrable and proved to be specific, they may be of use in diagnosis of vinyl chloride related liver disease.

Objective

1. To test for liver antigenic changes in a) angiosarcomatous liver tissue, b) chemically-induced hepatomas, and c) cultured human liver carcinoma cells.

2. To test for abnormal serum antigens and autoantibodies in patients with histories of vinyl chloride exposure including individuals with liver angiosarcoma, liver dysfunction with fibrosis, and with normal liver function tests. Antigens studied included liver specific antigen (LSA) (19), and F-antigen (20,21), tissue antigens of wide organ distribution (22,23), and bile antigens (24). Autoantibodies were to nuclei, mitochondria and smooth muscle. Patients' sera were also tested by immunofluorescence for possible reactivity with rats exposed to vinyl chloride (25).

Research Results

1. Liver antigenic changes

a) angiosarcomatous liver tissue

Liver angiosarcomatous tissue was analyzed in this work for presence of neoantigens, normal tissue antigens and tumor bound immunoglobulins. Several normal tissue antigens were found by immunodiffusion to be present in the tumor. These included the previously described liver-specific antigen, (LSA) (19) and antigens which are shared with several tissues (22,23). In addition, a tumor associated protein antigen and a glycoprotein liver antigen found to be absent in angiosarcoma were identified and characterized (25).

Tissue distribution of liver angiosarcoma related antigen is shown in Table 1. Preparations containing tumor associated antigen were found to inhibit lymphocyte transformation tests in vinyl chloride workers suggesting that these workers have a decreased lymphocyte response to these preparations (26). Further, antigen coagulation Factor VIII was detected in the angiosarcoma cells, pointing to an endothelial cell origin of the tumor (25). Finally, immunoglobulin G bound to the angiosarcomatous tissue was demonstrated by immunofluorescence and elution experiments suggesting antibody stimulation by the tumor (25).

TABLE 1

TISSUE DISTRIBUTION OF LIVER ANGIOSARCOMA-RELATED ANTIGEN

TISSUE EXTRACT USED AS ABSORBENT	ANGIOSARCOMA-RELATED ANTIGEN*
Liver angiosarcoma	+
Liver	-
Kidney	-
Spleen	+
Lung	+

Antiangiosarcoma serum was absorbed with lyophilized tissue extracts, 100 mg/ml, and tested against angiosarcoma extract.

*Presence of antigen indicated by +.

b. chemically-induced hepatomas

Our finding of an antigen missing in YC-related liver angiosarcoma stimulated further studies of antigenic deletion in chemically-induced hepatomas and in cultured human liver carcinoma cells.

In studies performed with the fast growing and undifferentiated chemically-induced Morris hepatoma 7777, 2 liver antigens were found to be absent. These antigens were characterized and partially isolated. In studies of their occurrence in other tissues, one of these antigens (Antigen I) was shown to be present in kidney and spleen in addition to liver. The second antigen (Antigen II) was detected only in liver. Antigen II was found unrelated to liver-specific F-antigen, differing in a number of properties and in immunologic reactivity.

In studies of their subcellular distribution in normal liver, Antigen I appeared localized in cytosol (54%) and mitochondrial (38%) fractions. Antigen II was about equally distributed in cytosol, mitochondria and nuclei fractions with little amounts in microsomes. Antigen I has a electrophoretic mobility in immunoelectrophoresis close to that of serum gamma-globulins and Antigen II to that of serum alpha-globulins. The two antigens were completely inactivated with Pronase indicating that both antigens are proteins or protein associated. Both antigens were relatively thermolabile; they were partially inactivated following incubation at 56°C and completely inactivated at higher temperatures. Both antigens were completely inactivated when incubated in pH buffer lower than 3.5. In Sephadex-G200 gel filtration, Antigen I behaved like a protein of approximately 51,000 Daltons, using as standards serum albumin, ovalbumin, chymotrypsinogen and ribonuclease. The molecular size of Antigen II (determined on a Bio-gel A5m column) was approximately 240,000 Daltons, using aldolase, catalase and ferritin as markers.

The two antigens were found in the more differentiated and slower growing hepatomas 5123tc and 9618A at about the same concentration as normal liver. The fact that hepatoma 7777 is the fastest growing and least differentiated of the tumors studied suggests a possible functional relationship between the absent antigens and these properties (28). These antigenic deletions may be used as indicators in the early detection of liver tumors and in the evaluation of the rate of growth, histologic differentiation and metastatic properties of such hepatomas.

Another liver constituent which may serve as a sensitive indicator of chemically-induced liver tumors, liver-specific F-antigen, was studied. In studies on the behavior of this antigen in Morris hepatomas, it was found that different types of these chemically-induced tumors have quite different levels of F-antigen.

F-antigen appeared to be absent in the fast growing hepatoma 7777. In the slow growing hepatoma 9618A, the concentration was very low ranging from less than 2% to 10% of the normal liver concentration. The medium growing hepatoma, 5123tc, highly metastatic, had about twice the concentration as

normal liver. F-antigen of hepatoma 5123tc and of normal liver were found localized in the cytosol subcellular fraction and were determined to be immunologically identical and to have equivalent electrophoretic mobility and molecular weight (29).

Since the antigen was undetectable in the fast growing hepatoma and undetectable or very low in the slow hepatoma, the level of F-antigen does not appear to correlate with the rate of growth of these tumors. A possible relationship between metastatic properties and F-antigen is now being considered because the hepatoma with the increased concentration of F-antigen was by far the most highly metastatic. This may prove useful in treatment of tumors.

c) cultured human hepatoma cells

In studies on cultured human liver carcinoma cells (after establishing optimal conditions required for the maintenance in serum free media of PLC/PRF/5 human liver carcinoma cells) it was determined that these hepatoma cells, similar to the experimental Morris hepatoma 7777, are deficient in liver-specific F-antigen. Nevertheless, these cells, like normal liver cells, produce serum albumin, fibrinogen, transferrin, alpha-1 antitrypsin and alpha-2 macroglobulin as shown by immunodiffusion (30,31) and immunofluorescence (32). These data add further support to the clinical observation that tissue antigens are more useful for treatment and follow-up care than screening and early detection, and that antigenic deletions may prove useful in early screening.

2. Abnormal Serum Antigens and Autoantibodies

Serum autoantibodies to nuclei, mitochondria and smooth muscle were negative in all patients examined. In addition, serum from these patients did not show reactivity with liver from rats exposed to vinyl chloride. Liver-specific antigen LSA (19), bile antigens (24) and other tissue antigens (22, 23) associated with liver damage were not detected in these patients (25).

Relevance to Industry

These studies are relevant to chemical industry in view of the evidence provided showing important changes in the levels of angiosarcomatous liver-antigenic components in association with vinyl chloride exposure. Deletion of liver antigens were also demonstrated in chemically-induced hepatoma. In addition, these observations may eventually prove useful in the development of new approaches for screening of chemical-associated tumors.

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PROGRAM H

THE USE OF ISOLATED MAMMALIAN LIVER CELLS FOR THE STUDY OF CHEMICAL MONOMER METABOLISM; Investigator, R. C. Feldhoff

H1. Isolation of Mammalian Liver Cells for the Study of the Metabolism of Chemical Monomers

H2. The *In Vitro* Study of Albumin Synthesis by Liver from Human Biopsies

Background

Presently there is no simple or even complex method to assess the functional capability of the human liver to handle exposure to various chemicals and/or their metabolites. In general, the rat has proven to be the most useful experimental animal for studying hepatic metabolism. Perfused liver slices and isolated hepatocyte systems have been developed to better study the interrelationship and to control the myriad processes of liver metabolism. In the perfused liver the architecture of the organ is preserved but separated from other body influences. Newer *in situ* techniques allow the liver tissue to never be without a supply of oxygenated red cells. The same technique can also be used to prepare isolated liver cells enzymatically.

The intact liver consists of at least four cell types, whereas isolated liver cells are nearly homogeneous in population. These cell suspensions provide technical advantages in that a number of conditions can be tested at one time and many uniform serial samples can be collected. Preliminary investigations were therefore done to determine whether the human liver biopsy material can function in a manner equivalent to liver slices and whether these tissues would demonstrate similar findings to those of isolated cell suspensions. If this technique were made functionally feasible it could then be used to study the metabolism of xenobiotics using viable human liver tissue in an *in vitro* manner.

Objective

1. To determine if an *in vitro* adaptation could be developed which would utilize animal/human biopsy material for assessing the functional capability of human liver cells. Protein metabolism has been used as the standard indicator and criteria for comparison

2. To determine the usefulness of this technique in human biopsy material obtained for routine medical purposes via percutaneous and transvenous approaches.

Research Results

Our first step was to verify the clinical and biochemical conditions needed for handling human liver biopsy material obtained during routine medical procedures. Appropriate assay conditions are being determined to assess the functional capability of the liver at the time of the biopsy to synthesize retained and secreted protein. At the same time, the biopsy's histopathology will be assessed and correlated with the biochemical data. The technique used to study both protein synthesis and secretion is shown in Figure 1. The histopathology is illustrated in Figure 2.

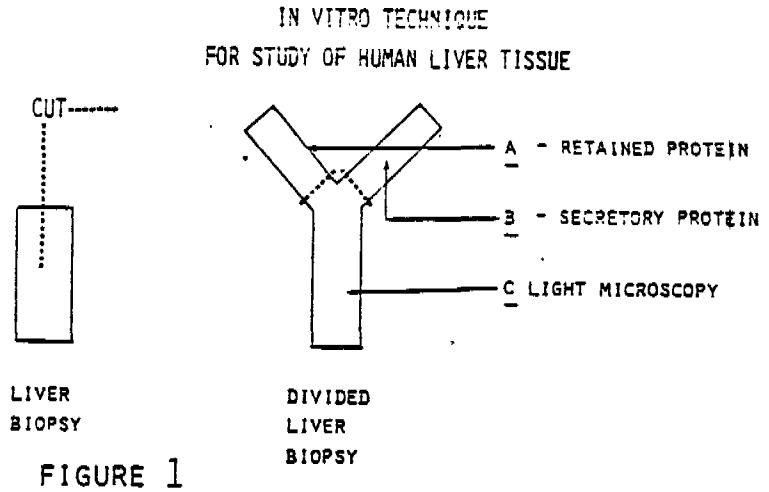

FIGURE 1

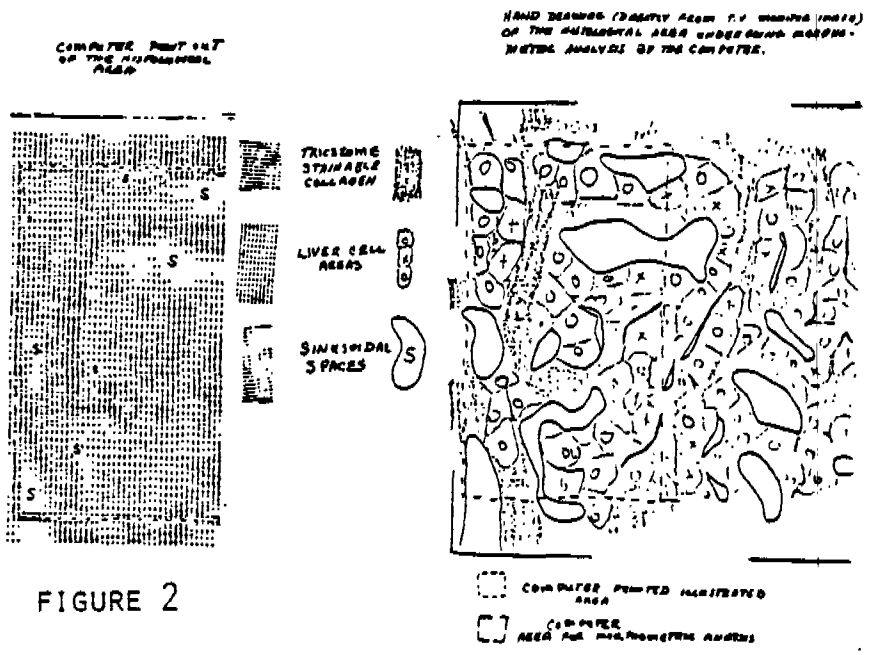

FIGURE 2

The initial experimental studies utilizing liver perfusion and the isolation of hepatocytes techniques provided the hepatocytic and nonparenchymal cells for Du and Tamburro's study of the oxidizing and detoxifying capabilities of these cells. Subsequent experiments have been used to quantitate the intracellular albumin synthesis, the rate of albumin secretion by the use of specific antibody fractions available for human and rat albumin. Initial results are illustrated in the rocket immunoelectrophoresis patterns from rat liver biopsy study (Figure 3) and the amino acid analysis is seen in Figure 4. The technique for liver cell identification after cell separation by the perfusion technique is illustrated in Figure 5.

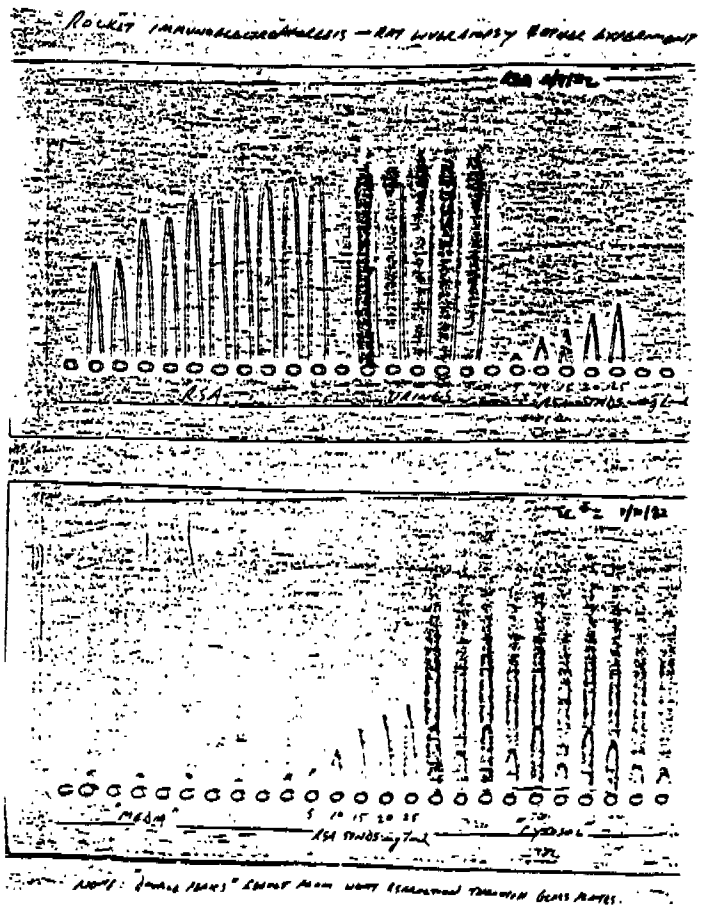

FIGURE 3

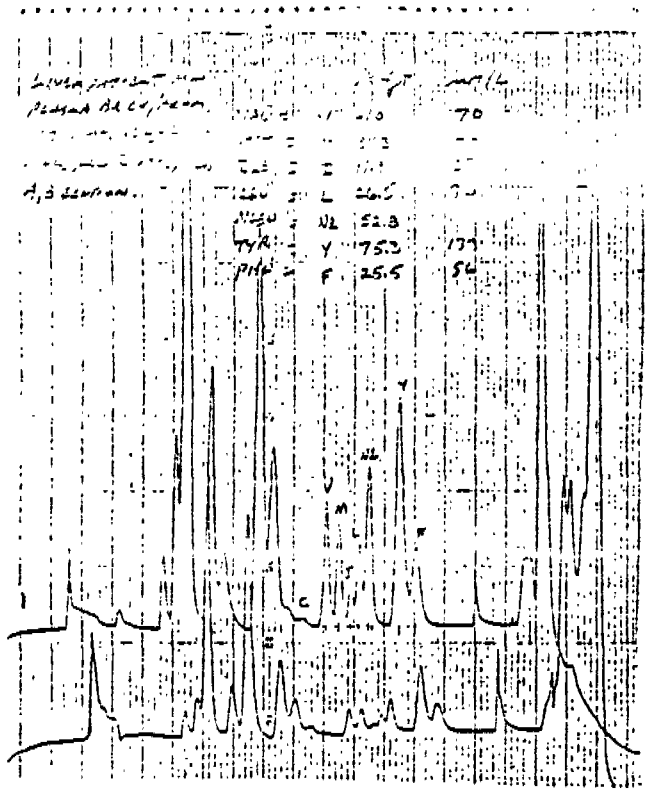

FIGURE 4

TECHNIQUE FOR HEPATIC
CELL TYPE IDENTIFICATION

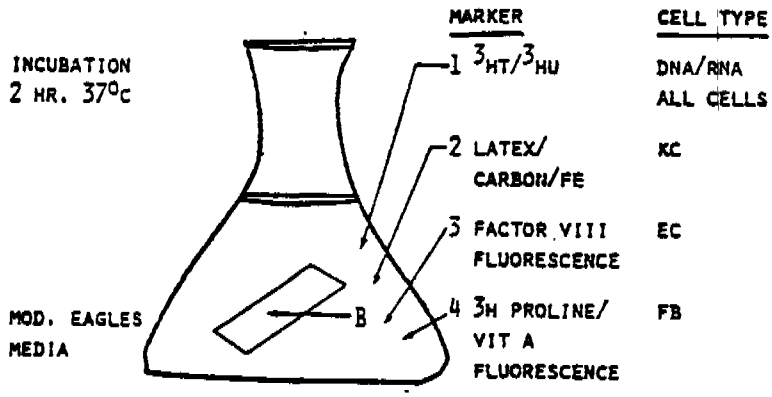

FIGURE 5

Relevance to Industry

Although this study was a last year addition to our initial proposal, it has provided the preliminary exploration into the adaptation of highly sophisticated laboratory techniques to human tissue in an *in vitro* system. While the work of these studies is still developmental, it provides promise for future clinical use. Such use could include determination of an individual liver's ability to oxidize and detoxify xenobiotics. The degree and nature of liver cellular injury or impairment could be determined in a quantitative biochemical fashion, and could be directly correlated with

morphological findings. These techniques could be also used to determine the degree of biochemical adaptations of the human tissue in individuals who have been exposed to xenobiotics. Finally, these methods could, on sequential biopsy, provide clinical information regarding whether these alterations persist or revert back to a normal state.

Although this is a very futuristic attempt to adapt presently developed methodology, it does accurately reflect the direction in which clinical research should be directed in order to better assess the human capability of sustaining environmental changes.

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PROGRAM I

THE STUDY OF TISSUE DISPOSITION OF INDUSTRIAL CHEMICALS: THE VINYL CHLORIDE EXAMPLE; Investigators - W.J. Waddell and C. Marlowe

II. The Use of Whole Body Autoradiography in Specific Tissues. Localization of Accumulated and Retained Industrial Chemicals and Their Metabolites

Background

Vinyl chloride has been classified by regulatory agencies as a carcinogen. Chronic exposure of workers to this carcinogen has resulted in angiosarcoma of the liver, as well as other tumors (Creech and Johnson, 1974). Several types of tumor also are observed after vinyl chloride exposure to animals (Maltoni and Lefemine, 1974). How or why these various body sites developed tumors was unclear. The need for methods which more accurately identify the sites of localization of chemical agents and their metabolites has obvious importance. The use of whole body autoradiography to identify the ultimate location of radioactively tagged chemicals was studied utilizing ^{14}C-vinyl chloride.

Objective

1. To determine the sites of localization and retention of the metabolites of vinyl chloride in mice.

2. Identification of the tissues of localization should help elucidate the mechanism of carcinogenic action of vinyl chloride.

Research Results

The sites of localization of radioactivity in tissues of the mouse following exposure to ^{14}C-vinyl chloride were studied by the technique of whole-body autoradiography (Waddell and Marlowe, 1977). Male CD-1 mice purchased from Charles River, weighing 25-31g., were exposed to ^{14}C-vinyl chloride in room air in a sealed chamber for 3 hours. The radioactive vinyl chloride was synthesized from ^{14}C-ethylene dichloride (New England Nuclear, Lot α1194-143, specific activity 3.2 mCi/mmole) immediately prior to exposure by the method of Wagner et al. (1975). The initial concentration of the vinyl chloride was 50 ppm with a specific activity of 1.84 mCi/mmole. At the end of the 3 hour exposure of the mice to ^{14}C-vinyl chloride the mice were removed to room air. Twenty minutes, 1, 3, 9 or 24 hours after removal of the mice to

[illegible] a mouse was briefly anesthesized with ether and sacrificed by freezing it in dry ice/hexane bath at -75°C.

The preparation of the radioactive vinyl chloride, exposure of the mice to this isotopic gas, and the sacrifice of the mice were done in collaboration with Dr. William T. Stott at Dow Chemical in Midland, Michigan. All remaining procedures were performed at the University of Louisville.

Whole-body sagittal sections of the mice, 20μ- and 40μ- thick were taken onto Scotch tape at -20°C. After freeze-drying, these sections were placed against Kodak AA X-ray film and allowed to expose in light-tight containers at -14°C for 6 to 39 weeks. The sites of localization of all nonvolatile metabolites of vinyl chloride were visualized in the developed X-ray film (autoradiograph) and reveals the in vivo disposition of these metabolites at the time of sacrifice of the mouse. These procedures for whole-body autoradiography, first described by Ullberg in 1954 do not allow thawing or contact with any solvents. Thus, there is no translocation nor loss of radioactivity. A complete description of these procedures have been published previously (Waddell and Marlowe, 1977).

Representative autoradiographs from each mouse were used as negatives to produce the prints included with this report. Therefore, white areas in the prints represent the sites of radioactive accumulation of the nonvolatile metabolites of ^{14}C-vinyl chloride.

The highest levels of radioactivity in the mice sacrificed 20 minutes and 1 hour after removal from the ^{14}C-vinyl chloride environment were observed in liver, pancreas, kidney, intestinal contents, urine and bile (Figures 1 and 2). Additional sites of localization of radioactivity in the 1-hour animal were thymus, thyroid and seromucous glands (Figure 2).

The concentration of metabolites in the organs of excretion decreased continually over the 24 hour period (Figures 3-5). By 9 hours after removal from the vinyl chloride, thymus, Harder's gland, epithelium of the esophagus and intestine, urine and sublingual gland retain the highest levels of radioactivity; moderate concentrations of radioactivity were observed in liver, kidney and intestinal contents (Figure 4). After 24 hours the primary organs of retentions of radioactivity were thymus and Harder's gland; some radioactivity could be visualized in liver and in esophageal and intestinal epithelium (Figure 5). Figure 6 shows the relative concentration of nonvolatile metabolites in the cortex and medulla of the thymus at the earliest and latest time intervals studied. Twenty minutes after removal from the vinyl chloride, the concentration of radioactivity in the thymus was only slightly increased above that of blood (Figure 1 and 6); however, by 1 hour the thymus showed the highest uptake of radioactivity in the body (Figure 2) and remained the highest throughout the 24 hour period (Figures 2 - 6).

^{14}C-VINYL CHLORIDE; 20 MIN AFTER REMOVAL

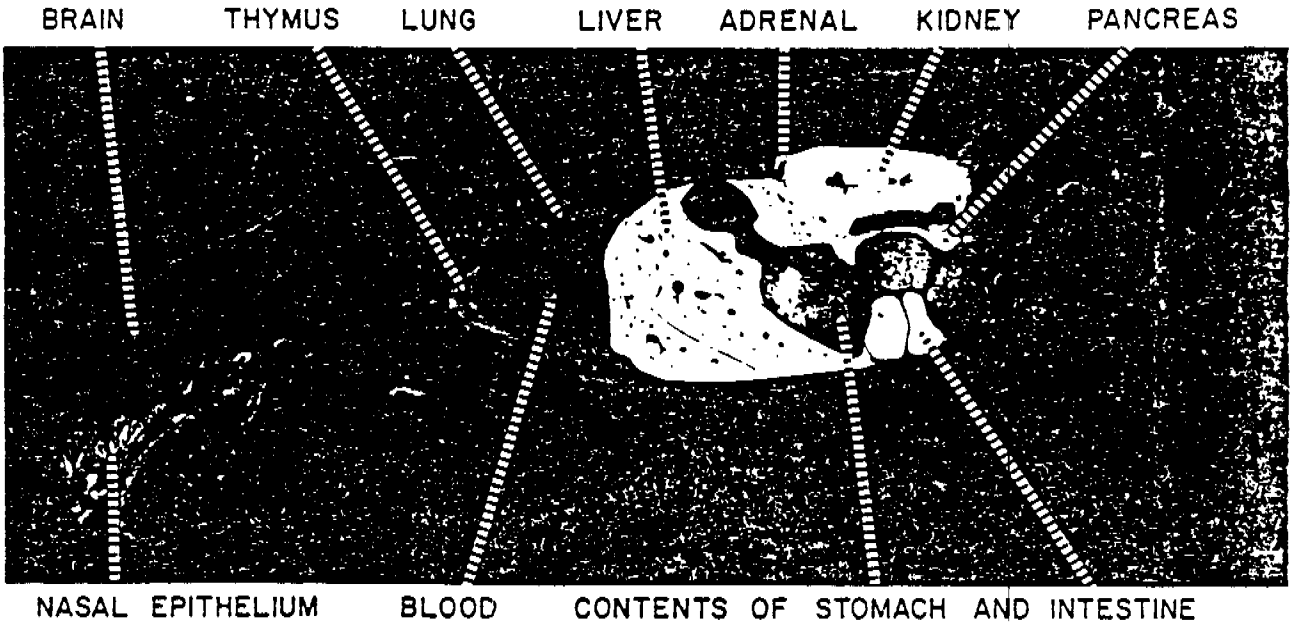

Figure 1: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 20 minutes after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE; 1 HR AFTER REMOVAL

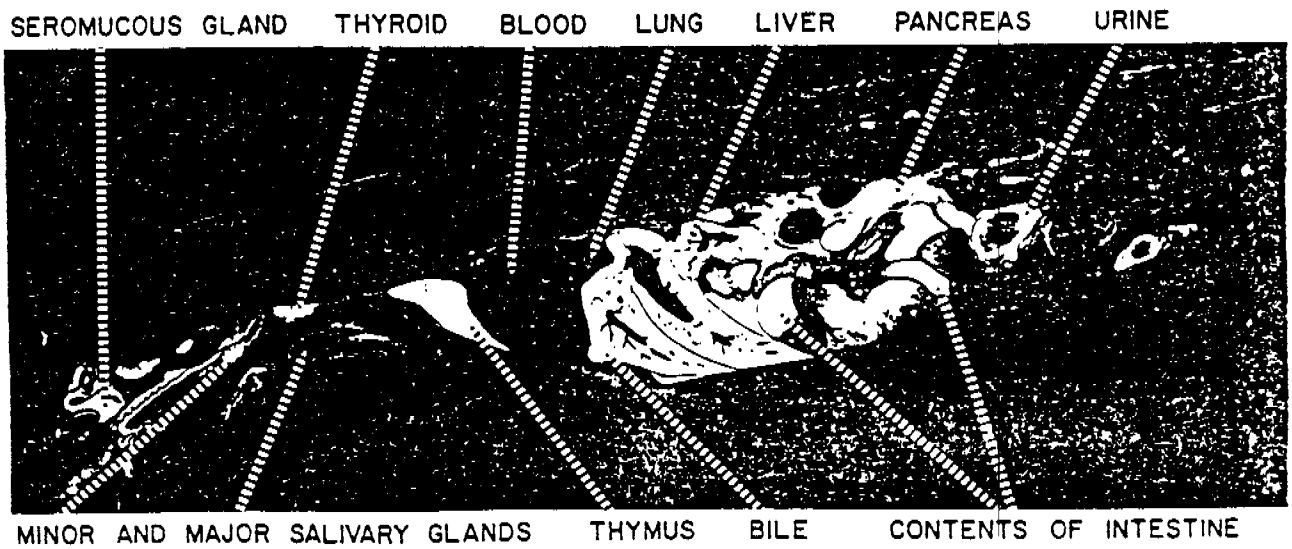

Figure 2: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 1 hour after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE; 3 HR AFTER REMOVAL

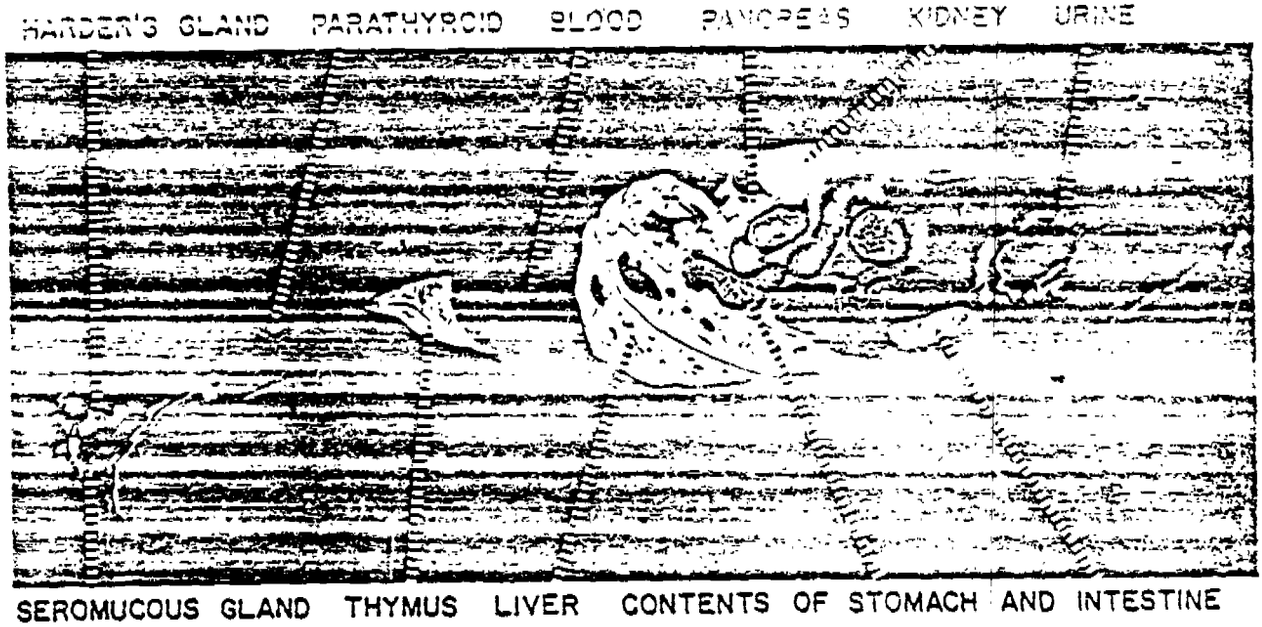

Figure 3: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 3 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE; 9 HR AFTER REMOVAL

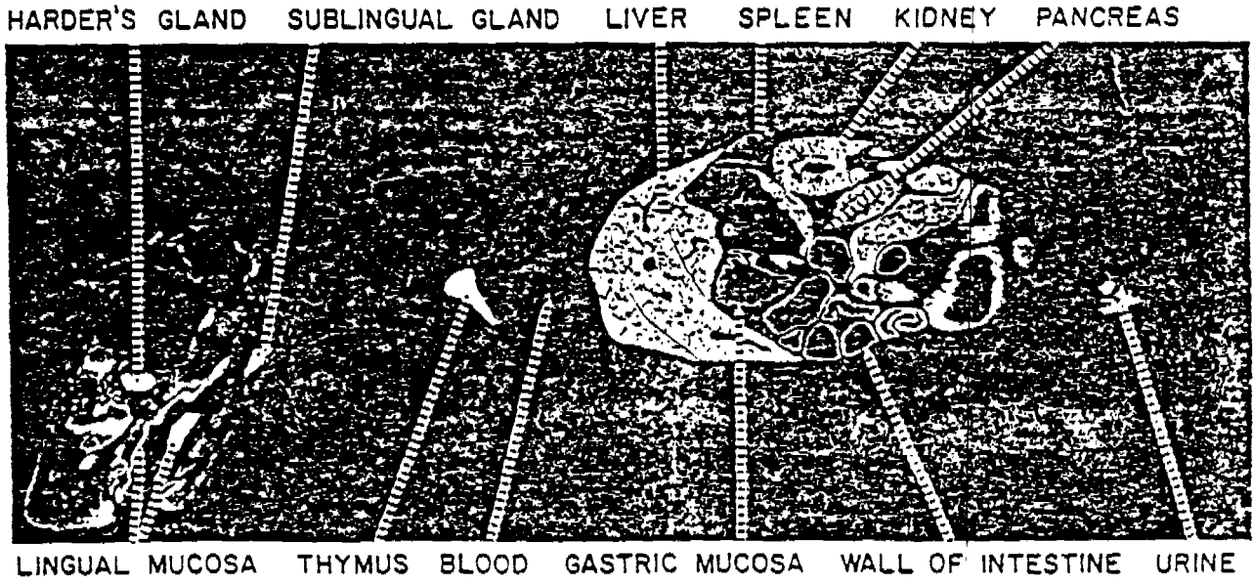

Figure 4: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 9 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE; 24 HR AFTER REMOVAL

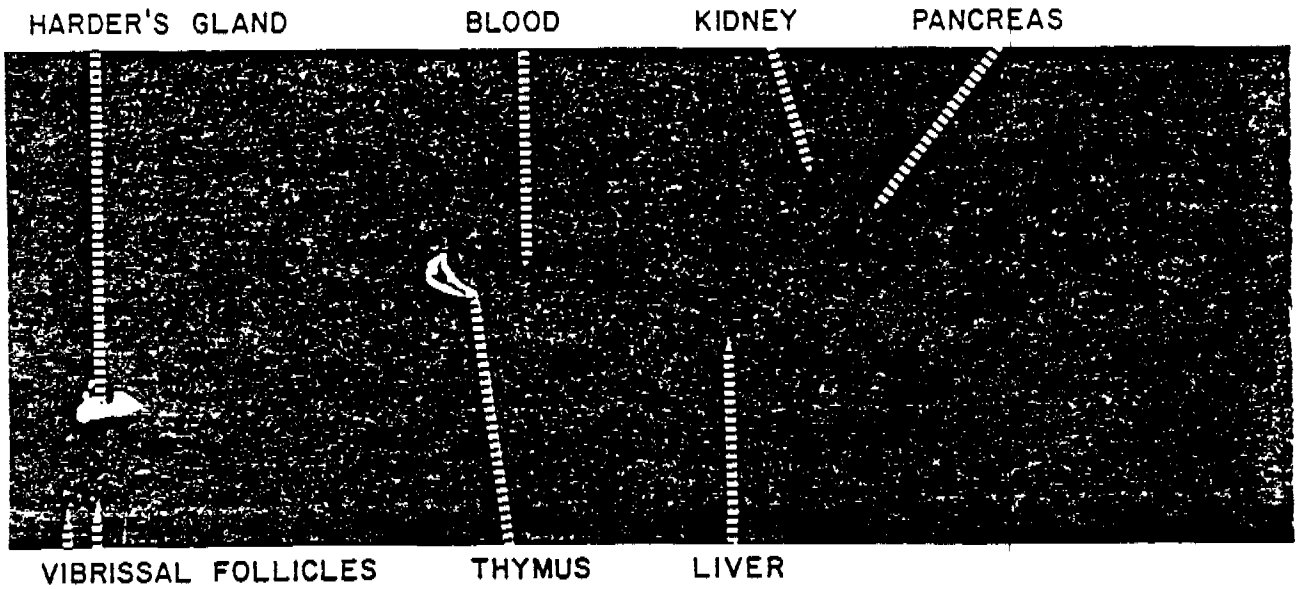

Figure 5: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 24 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE

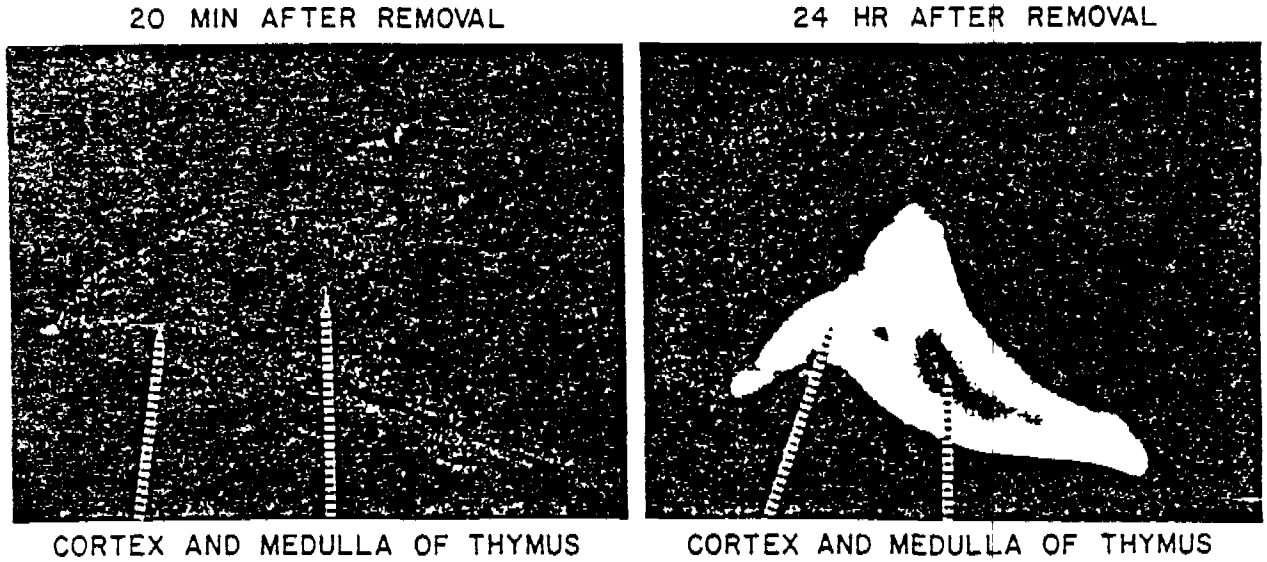

Figure 6: Prints of the thymus areas of whole-body autoradiographs from male CD-1 mice which were exposed to ^{14}C-vinyl chloride for 3 hours and then frozen 20 minutes or 24 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity. Note the high retention of the nonvolatile metabolites in the cortex of the thymus after 24 hours.

The sites of localization of the nonvolatile metabolites of vinyl chloride in the mouse are similar to those reported for the rat (Duprat et al. 1977). The high concentration of nonvolatile metabolites of vinyl chloride retained in the thymus after 24 hours suggests that there is covalent binding of these metabolites to molecules in the thymus. It is possible that these molecular interactions in the thymus could have an effect on the immune system. The high concentration in the thymus may be involved in the stimulation of the immune system in mice seen by Sharma and Gehring (1979) and in the reduction in peripheral T-lymphocytes seen in man by Ward et al. (1976).

Vinyl chloride may exert its carcinogenic action by a dual mechanism. It may suppress the immune surveillance system mediated by the thymus and concurrently damage several tissues including the liver.

Relevance to Industry

Whole-body autoradiographic studies on the biological disposition of industrial chemicals offers the most thorough approach to the study of the interaction of these chemicals with tissues in the body. Knowledge of these tissue interactions allows predictions of potential toxicity or, conversely, to a lack of toxic effect if the chemical is rapidly and completely eliminated (Waddell et al., 1977). When chemicals are retained in specific tissues as in the case with vinyl chloride, the tissue of retention gives an indication of possible mechanisms of toxic action. Vinyl chloride appears to be having a toxic effect on the immune system; the direct evidence for an interaction of the chemical with this system was not available until these studies were done.

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LISTS OF PUBLICATIONS, ABSTRACTS, PREPRINTS AND PUBLICATIONS IN PREPARATION

PUBLICATIONS

1. Fortwengler, Jr., H.P., Jones, D., Espinsoa, E. and Tamburro, C.H. (1981) Evidence of Endothelial Cell Origin of Vinyl Chloride-Induced Hepatic Angiosarcoma. Gastroenterology, 80:1415-1419.

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