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SCHOOL OF MEDICINE
DEPARTMENT OF MEDICINE
DIGESTIVE DISEASES AND NUTRITION SECTION

HEALTH SCIENCE CENTER
WALNUT & PRESTON STREETS

April 11, 1977

Mr. George E. Best
Vice President, Secretary-Treasurer
Manufacturing Chemists Association, Inc.
1825 Connecticut Avenue, N.W.
Washington, D.C. 20009

Dear Mr. Best:

RE: Initial Progress Report for the Manufacturing Chemists Association's Agreement with the University of Louisville

The following describes the initial progress during the first months of the Manufacturing Chemists Association's agreement with the University of Louisville entitled "Research Techniques and Methods for Detection and Prevention of Carcinogenesis in Industrial Workers." The technical proposal is composed of seven parts. Progress for each part will be reported separately.

Technical Proposal A - Immunological Systems for the Detection of Vinyl Chloride and Other Chemical Injury

Part 1

Fortwengler and group have been evaluating three independent aspects of the immune system of industrial workers exposed to vinyl chloride to determine whether these various functions of the immunological system can be used for the detection of future cancer development and whether alternative systems may be able to identify high-risk groups. A study of a subpopulation of lymphocytes (T cells) that are responsible for eliminating foreign growths and tumors from the body is underway. Their total number and ability to function have already been studied in 70 individuals with various degrees of exposure to vinyl chloride. This group is being compared to normal controls, using a panel of 10 standard immunological tests in an effort to determine a pattern of depressed immunity which may lead to the early detection of vinyl chloride injury and possible future development of angiosarcoma or other tumors. The recent updating of work and exposure histories on these workers (which was completed March 20, 1977) will now allow us to begin analysis and comparison between the exposed group and the control groups.

Part 2

The seeking of genetic markers using the histocompatibility tissue antigens (HLA) in order to determine an increased association with the development of vinyl chloride injury and angiosarcoma has gotten well underway. Tissue-typing has been completed on approximately 100 individuals with and without vinyl chloride liver injury and with varying degrees of vinyl chloride exposure. Frequency analysis will be performed after approximately 250 individuals have been completely typed. A recent finding by other investigators that an increased frequency of HLA-B27 in another industrial disease--Asbestosis--leads us to believe that our frequency studies may be able to predict which individuals have increased risk of vinyl chloride exposure. Work is continuing in this endeavor.

Part 3

In an effort to capitalize on the well-established fact that the body may mount an immune reaction to cancer, the Fortwengler group is endeavoring to develop a relatively specific test for angiosarcoma. Lymphocytes (the cells responsible for one's immunity) are being isolated and grown in the presence of a liver extract prepared from both normal individuals and individuals with angiosarcoma. In the early stages of tumor development, human lymphocytes may develop sensitization to various protein markers on the tumor and, when incubated in the presence of similar antigenic material, transform; i.e., change morphology and proliferate. This transformation can be measured quantitatively and acts as an indicator of early cancer development. This test under development could be performed on blood specimens in the laboratory and could indicate in a manner analogous to the immunological test for tuberculosis that a person either has angiosarcoma or has been sensitized (exposed) to it. About 60 individuals have been tested thus far--3 of which have given elevated reactions. One of these 3 positive reacting individuals was a patient with angiosarcoma. Two others had very high exposure histories to vinyl chloride. Further checks for specificity are in progress.

Technical Proposal B - Biochemical Enzymatic Systems for the Detection of Vinyl Chloride and Other Chemical Injury and Cancer Development in Industrial Workers

Part 1: Liver Specific Enzymes

Du and group have begun an evaluation of sorbital dehydrogenase, a liver specific enzyme. Present available enzymatic studies of liver function--SGOT, SGPT, LDH, etc.--are non-specific enzymes which can be elevated due to other organ injury. The need to identify the source of these common enzyme studies has led to the evaluation of

sorbital dehydrogenase as a specific marker of liver injury. Twelve hundred serum specimens for sorbital dehydrogenase activity have been determined. Analysis for comparison with standard enzymatic studies and histological documentation of injury is now underway. Verification of its specificity and determination of its sensitivity will then lead to an analysis of its ability to detect injury from exposure to other chemicals as well as vinyl chloride.

Part 2: Bile Acid Clearances

At present, the most sensitive diagnostic test for hepatocellular injury due to vinyl chloride exposure is indocyanine green clearance. This anionic dye is very sensitive to abnormalities in liver function and injury. It is, however, a foreign material. It has the potentiality for developing allergic reactions (11 of which have been noted in 2,000 determinations--this is mainly mild itching and transient rash) and is very costly to perform (material alone is over $100 per test). The need for an equally sensitive clearance study using a natural biological material at lower cost is evident. Bile acids of normal biological product cleared by the liver are under evaluation. Some 80 bile acid clearance studies have been performed. Analysis and comparison with ICG clearances for both sensitivity and specificity will begin shortly.

Part 3: Animal Studies

Animal studies have been conducted to determine the enzymatic changes in early vinyl chloride exposure. This covers a period of 14 to 140 hours of exposure in a total period of two to four weeks. Three specific enzymatic alterations in liver have been demonstrated to occur in early exposure periods without evidences of conventional clinical biochemical abnormalities. These alterations have included a decrease in glucose-6-phosphatase, an increase in glucose-6-phosphate dehydrogenase, and an increase in glutathione reductase. All three enzymatic changes have also been reported to occur in primary hepatocellular cancers of the same animal species. We believe that these changes reflect the adaptation of the liver cell to chronic or recurrent exposure to this chemical and probably predisposes the cell to ultimate cancer transformation. Phase II is now underway to determine whether enzyme changes related to nucleic acid synthesis will begin to occur as it does in the primary hepatocellular tumor. If future enzymatic studies continue to verify this progression of cellular activity, we would undertake the development of clinically applicable means of determining these changes as an indicator of excessive vinyl chloride exposure.

The elevation in glutathione reductase appears at the moment to be the most promising lead since this enzyme may be determined in peripheral blood, and its elevation may serve as an indicator of excessive vinyl chloride exposure and metabolism. Its specificity for chemical detoxification must, however, be verified. Studies are under design to determine this.

Technical Proposal C - Glycosaminoglycan Changes in the Early Detection of Fibrotic Injury and Hepatic Cancer

Kupchella, et al., have pursued the observation that tumors are associated with an elevation in tissue glycosaminoglycans (GAG). They have already establishd that angiosarcoma is included in this group of tumors. Secondly, it is known that GAGs are also found in normal connective tissue synthesis and scar formation and are elevated in connective tissue disorders. Since angiosarcoma is both a neoplasm and is associated with fibrogenesis, it was anticipated that the GAG changes could serve as signals for early lesions and might be useful in evaluating the progression of chemical injury to the liver before cancer transformation. The scope of this project falls within three categories:

(1) The determination of the usefulness of urinary and/or serum GAG patterns as an index of hepatotoxicity leading to fibrosis secondary to vinyl chloride exposure.

(2) To develop an efficient, simple procedure for determining the urinary and/or serum GAGs for use in routine medical surveillance and screening.

(3) To further characterize the role or roles of GAG in hepatic fibrosis and cancer development.

Preliminary Studies

Initial studies, begun with the support from the B. F. Goodrich Company and continued with this grant support, have included:

(1) Histochemical evaluation of angiosarcoma of the liver demonstrating an increase in GAG in the tumor and in hepatic tissue adjacent to the angiosarcoma;

(2) Biochemical GAG analysis of the angiosarcoma tissue itself also showing significant elevation in the GAG;

(3) An evaluation of 50 human urine specimens from cases of angiosarcoma, cirrhosis, chronic hepatitis, and metastatic cancer to the liver showing elevated urinary levels of GAG;

(4) Chromatographic evaluation of the urinary GAGs in angiosarcoma in both early and advancing stages demonstrating a progressive shift from the hyaluronidase-susceptible GAG fraction to the hyaluronidase-resistant GAG fraction;

(5) Studies using the rat carbon tetrachloride-induced fibrosis model demonstrating increases in both tissue and urinary glycosaminoglycans.

Studies in Progress

(1) Evaluation of daily GAG variation in normal urines and angiosarcomanous urines;

(2) More complete evaluation of the procedure used for assaying urinary GAGs, including recovery and reproducibility studies;

(3) Evaluation of early urinary and tissue GAG changes caused by carbon tetrachloride exposure; and

(4) A comparison of urinary and tissue GAG constituents in:

(a) the urine and tissues of normal rats,

(b) the urine and tissues of rats in which fibrosis has been induced by carbon tetrachloride,

(c) the urine and tissues of normal humans and those from the cases of angiosarcoma and primary hepatocellular cancer.

Technical Proposal D - Histological Systems of Detection

Schrodt and Tamburro are continuing their analysis of light microscopic and electron microscopic findings of liver biopsy tissue obtained from B. F. Goodrich workers with and without vinyl chloride exposure. Eighty (80) biopsies are being studied for light microscopy and sixty (60) for electronic microscopy. In several cases, sequential biopsies have been obtained for both light and electronic microscopy study. Seven (7) different biopsies have been studied by electron microscopy, and over twenty-five (25) sections have been characterized as to fibrous content and its location. Determination of sinusoidal size and cell changes has just begun with the arrival of a calculator-digitizer. This instrument will assist in

morphometric analysis of the ultra structures and histological findings and should speed up considerably the time required normally by the manual technique. The computer program for the morphometric analysis of the specimens is near completion.

After completion of the initial 20 individual biopsies' evaluation (both by electron and light microscopy), statistical analysis will begin to compare these findings with work history exposures to vinyl chloride and other chemicals.

Technical Proposal E - Chemical Systems of Detection

Wong and group have already synthesized vinyl chloride and its metabolites (chlorooxirane, chloroacetaldehyde, chloroethanol and chloroacetic acid). In cooperation with Dr. Streips, they have demonstrated the mutagenicity of two of vinyl chloride's metabolites--chlorooxirane and chloroacetaldehyde. Studies have continued during this initial period to further elucidate the key intermediates in the metabolic conversion of vinyl chloride.

The formation of 2-chloroethanol from vinyl chloride via chloro-oxirane (a diradical intermediate) has been demonstrated. These studies have explained the unknown pathway leading to chloroethanol formation which is a key metabolite in the biological conversion of vinyl chloride.

The mechanism of deactivation of the chloroacetaldehyde by cysteine has also been studied. The derived cyclic product--3L-carboxy-2, 3-dihydro-1, 4-thiazine--may be the precursor of urinary metabolites S-hydroxyethyl-cysteine. Further, they have confirmed that chloro-ethanol and chloroacetic acids do not react with cysteine at physiological pH's.

These studies, which further elucidate the intermediary metabolism of vinyl chloride, are initial steps in better understanding the metabolic activation and inactivation of vinyl chloride. This is essential in being able to properly determine which end products or intermediates would be the best products to analyze in body fluids and tissues by gas chromatography and mass spectrometry by quantitating the metabolic end products of vinyl chloride and establish at which levels of exposure these products develop, as well as their concentration and distribution in various body fluids and tissues in animals. Validation of these studies in animals will then be applied to human materials. The first target metabolite for analysis is chloroacetic acid. The methodology for this is now under development.

Technical Proposal F - Assays for the Carcinogenic Potential of Industrial Chemicals Utilizing Prokaryotic and Eukaryotic Systems

Streips and group have focused their efforts in the first reporting period at two major areas:

(a) The further elucidation of the molecular mechanisms involved in vinyl chloride-mediated mutagenesis --

They have previously determined that the post replication repair (an error-prone DNA repair process) is used in rectifying chloroacetaldehyde--the proposed mutagenic metabolite of vinyl chloride--induced DNA damage. Post replication repair alone has at least seven unique deficient mutants. Their previous work only used one, and they have expanded their screening system to include all available repair deficient strains. The results of these experiments are shown in Table 1. Their preliminary studies using chloroacetaldehyde have allowed them to separate the available DNA repair mutants into two groups: Those which are affected by chloroacetaldehyde and those which are not. These studies open the possibility of investigating specific gene products that are necessary for repairing chloroacetaldehyde damage and then relating these to similar products in mammalian cells, with the possibility in the future of applying this to human cells. These systems have used backward mutation analysis.

(b) Screening for potentially harmful industrial and chemically relevant chemicals --

To facilitate their screening procedures, they have instituted an additional assay--the forward mutation analysis. This test measures directly the capability of a chemical to cause a mutation in a cell. Similar assays can be performed in mammalian cells. The results of these assays, showing the capability of chloroacetaldehyde to cause forward mutations at a high frequency and comparing them to mutation capacities of methyl methane sulfonate (a potent alkylating agent and carcinogen), are shown in Tables 2 and 3. These data indicate that all their strains are prone to both chloroacetaldehyde and methyl methane sulfonate for mutagenesis.

Thus, this laboratory now has the availability of the following screening procedures:

(1) The salmonella reversion assay, which measures base substitutions and deletions.

(2) The bacillus repair assay, which measures the susceptibility deficient strains to chemicals.

(3) The forward mutation rate assay.

Using these tests, they have examined several samples for mutagenic activity, as shown in Table 4. Future studies will include other selected chemicals used in chemical industry, a few examples of which are listed in Table 5.

Technical Proposal G - Tissue Antigens and Antibodies in the Detection of Vinyl Chloride Injury

Espinosa and group, in their search for markers of liver injury associated with vinyl chloride exposure, have so far made the following observations. In human liver angiosarcoma, a tumor-associated antigen has been demonstrated by immunodiffusion procedures. This was characterized as a protein, inactivated by Pronase and Trypsin, relatively susceptible to heating and acid pH, and precipitated mainly at 20 to 30 percent ethanol concentration and at ammonium sulfate saturation of 30 to 70 percent.

Additional observations were made that the angiosarcoma tumor did not have an antigen normally found in the liver. This particular liver antigen has properties of a glycoprotein, since it is inactivated by periodate treatment and is unaffected by Pronase and Trypsin. In addition, it is relatively thermostable, affected by acid pH and precipitated over a wide range of ammonium sulfate and ethanol concentrations. Further studies to both characterize the angiosarcoma tumor-associated antigen, as well as further studies of the absence of normal liver antigens, are continuing in order to determine how the absence or presence of certain antigens identified by antibody studies can be utilized in the early detection of vinyl chloride injury and tumor formation. Cross studies with Fortwengler's group have begun to determine if the angiosarcoma tumor-associated antigen is present in the tumor extract used to stimulate human lymphocytes. Documentation of this would help to increase the specificity of the extract test for angiosarcoma tumor antigens and sensitized human white cells.

In addition, study of the human angiosarcoma has demonstrated by immunofluorescence and elution experiments that angiosarcoma has bound immunoglobulin G, suggesting antibody stimulation by the tumor. This may be another means of detecting the presence of angiosarcoma. Experiments to elucidate the possible value of this antibody in the detection of the angiosarcoma are now underway.

Finally, during this work process a new liver specific antigen was also demonstrated and is being characterized and evaluated in vinyl chloride-associated liver injury.

This completes the first Progress Report of the Manufacturing Chemists Association - University of Louisville Cancer Center agreement, which was due January 1, 1977. I apologize for the delay in the report--a combination of some administrative delays in signing of the agreement (May-October, 1976); and the severe winter snows in January and February interfered with our administrative efficiency, although research continued unabated. The next report due May 1, 1977, will arrive promptly. If there is need for any further information or questions, please feel at liberty to contact me.

Sincerely yours,

Carlo H. Tamburro MD

Carlo H. Tamburro, M.D.
Associate Professor of Medicine
Principal Investigator

CHT:pz

Enclosures

TABLE 1

REPAIR ASSAYS WITH B. SUBTILIS*

Strains	Average inhibition in mm/8 experiments
REC A	
recA1	13.5
recA1	7.5
REC B	
recB6	13.5
recB3	9.5
recB19	16.5
recB2	16.5
recB2	11.3
recB2	16.8
REC C	
recC7	NR
REC D	
recD27	11.5
recD27	NR
REC E	
recE61	6.7
recE4	8.8
REC F	
recF7	6.1
recF18	7.4
recF15	7.5
recF15	8.42
recF16	3.1

Strains	Average inhibition in mm/8 experiments
REC H	
recH342	NR
REC	
rec-4	15.4
rec-13	5.4
MTC	
mtc-41 (caf^r)	NR
UVR	
uvr-35 (caf^r)	NR
hcr	NR
uvr	NR
WT	
wt	NR
wt	NR
wt	NR

*100mM chloroacetaldehyde used in all these experiments

TABLE 2

INDUCTION OF STREPTOMYCIN RESISTANCE MUTANTS FOLLOWING 15 MINUTE CAA EXPOSURE

B. subtilis strains	CAA treatment	Total No. of bacteria O/N	Mutant Col. per Strept plate[1]	Mutant frequency per 10^8 bacteria	Relative mutations frequency[2]
168 WT	0	4.36×10^8	11.6	2.6	13.6
168 WT	5mM	3.2×10^8	113	35.3	
Hcr-9	0	4.85×10^8	7	1.4	14.2
Hcr-9	5mM	3.45×10^8	69	20.0	
MC-1	0	2.35×10^8	2	0.8	6.3
MC-1	5mM	0.65×10^8	3.3	5.07	

[1] 1 mg per ml dihydrostreptomycin sulfate in incubation media; 2 days of incubation

[2] Ratio = $\frac{\text{treated cells (mutation frequency)}}{\text{control untreated cells (mutation frequency)}}$

TABLE 3

B. subtilis strains	Total No. bacteria	Mutant colonies (Str^r)[1]	Mutation frequency per 10^8 cells	Relative mutation frequency[2]
168 WT	5.7×10^8	2.4	0.4	
168 WT + CAA(5mM)	5.3×10^8	70.8	13.4	33.5
168 WT + MMS(5mM)	5.6×10^8	85.8	15.3	38.3
Hcr-9	4.2×10^8	2.8	0.7	
Hcr-9 + CAA(5mM)	3.6×10^8	15.2	4.2	6.0
Hcr-9 + MMS(5mM)	5.1×10^8	19.8	3.9	5.6
Mc-1	2.0×10^8	1.8	0.9	
Mc-1 + CAA(5mM)	0.8×10^8	1.2	1.5	1.7

[1] 1 mg per ml dihydrostreptomycin sulfate in incubation medium (2 days of incubation)

[2] Ratio: $= \frac{\text{treated cells (mutation frequency)}}{\text{control, untreated cells (mutation frequency)}}$

TABLE 4

SUMMARY OF PERTINENT SUBSTANCES TESTED FOR MUTAGENICITY

		Salmonella	Subtilis
1.	Meta-chlorobenzoyl-cyclobutanecarbonyl peroxide	±	NR
2.	Benzoyl peroxide	NR	NR
3.	N-acetoxy-N-phenylacetamide	NR	NR
4.	N-cyclobutanecarboxyloxy-N-phenylacetamide	NR	NR
5.	bis-cyclobutane 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.	Chlorooxirane	+++	++
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

TABLE 5

LIST OF SELECTED CHEMICALS FOR MUTAGENIC STUDY

Acrylic Acid

Acrylamides--acrylamide, methyl, n-octyl, nMA

Acrylonitrile

Acetylene

Acrylates--ethyl, methyl, methyl-meth,
2 ethyl hexyl, n-butyl

Bisphenol A

Butadiene

Caprylyl chloride

Chlorinated solvents--carbon tetrachloride,
chloroform, trichloroethylene, EDC

Chloro ethyl vinyl ether

Diethyl maleate

Mecuric chloride

Methanol

Phenol

Toluene

Vinylidene chloride

Vinyl acetate

PVC dust

Catalysts

Styrene

Hexane