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ENVIRONMENTAL SCIENCES DIVISION

BIOLOGICAL (MOLECULAR AND CELLULAR) MARKERS OF TOXICITY

Semi-annual Technical Progress Report (No. 4)
April 1, 1990 to September 30, 1990

Lee R. Shugart

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Contract Title:

BIOLOGICAL (MOLECULAR AND CELLULAR) MARKERS OF TOXICITY

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Fort Detrick, MD 21701-5010

I. PURPOSE AND SCOPE OF RESEARCH EFFORT

The overall objective of this study is to evaluate the use of the small aquarium fish, Japanese Medaka (Oryzias latipes), as a predictor of potential genotoxicity following exposure to carcinogens. This will be accomplished by quantitatively investigating the early molecular events associated with genotoxicity of various tissues of Medaka subsequent to exposure of the organism to several known carcinogens, such as diethylnitrosamine (DEN) and benzo[a]pyrene (BaP).

The Japanese medaka has recently been used as a bioassay model to screen for carcinogenic compounds in water. The primary endpoint for most small fish carcinogenesis studies is the histopathogenic identification of a neoplastic lesion. Such lesions occur mainly in the liver, although other tissues in which lesions have been observed include nervous tissue, kidney, mesenchymal tissue, skeletal and smooth muscle. The histogenesis of liver neoplasms in fish is similar to that in rodents. Following exposure to chemical carcinogens, preneoplastic lesions appear as eosinophilic foci, basophilic foci, followed by tumor formation (Aquatic Toxicol. 11:113-128;1988). This is identical to the process that appears to occur in rodents and other mammals (Environ. Health Perspec. 75:65070;1987).

Therefore, if we are to understand in any depth the processes by which environmental chemicals exhibit genotoxicity, new and innovative techniques will be needed that elucidate the fundamental cellular nature of the various steps subsequent to exposure to these agents.

Because of the often long latent period between initial contact with certain chemical and physical agents in our environment and subsequent expression of deleterious health or ecological impact, the development of sensitive methods for detecting and estimating early exposure is needed so that necessary interventions can ensue. A promising biological endpoint for detecting early exposure to damaging chemicals is the interaction of these compounds with cellular macromolecules such as Deoxyribonucleic acids (DNA). This biological endpoint assumes significance because it can be one of the critical early events leading eventually to adverse effects (neoplasia) in the exposed organism.

The research will proceed along several interrelated lines of investigation, which have recently been reevaluated and revised:

Task 1. Evaluate current analytical techniques and

methodologies for their effectiveness to detect and quantitate biological responses at the molecular level to the action of genotoxic agents. Particular emphasis will be placed on of the adaptation of these methods to the detection of enzyme activities associated with detoxication and damaging events that occur to the DNA in the liver;

Task 2. Develop protocols for the exposure of Medaka under controlled laboratory conditions to DEN, AAF, and BaP and investigate the effects these chemical carcinogens have on the suite of molecular markers selected;

Task 3. Evaluate the short-term responses of the molecular markers as suitable biomarkers to estimate exposure and predict cellular effects to genotoxic chemicals.

The work proposed here focuses on the more fundamental aspects of the problems that deal with the detection of exposure to genotoxic agents through the appropriate use of molecular markers and the potential to predict subsequent adverse effects. Many chemical carcinogens and mutagens have the capacity to cause various types of DNA damage as a result of the interaction of highly reactive metabolites with DNA. Such interactions can form stable adducts with DNA, adducts which result in the formation of alkali-labile apurinic sites, and unstable adducts which cause strand breaks (American Sci. 70:386-393;1982). Indirectly, the fidelity of post replicative modification of DNA (such as minor nucleotide composition) can be affected by genotoxic agents. Each type of damage to the integrity of the DNA, if left uncorrected, could trigger a sequence of events that culminates in the appearance of an overt malignancy. Estimates of these various types of damage to DNA, along with an appraisal of the organism capability to maintain the integrity of it's DNA (repair), will provide the basis for determining bioavailability of the genotoxic agent and for estimating exposure. In addition the information may be used as a short-term predictor of the potential for long-term deleterious effects.

II. OVERALL PROGRESS

1. Task 1.

The purpose of this task is to evaluate the feasibility of using currently existing analytical techniques to detect molecular markers of genotoxicity, and included: (a) enzymes of detoxication and, (b) changes to the integrity of DNA. This task has essentially been completed and was reported in detail in Semi-Annual Technical Progress Report No.1 (ORNL/M-829, dated April 1, 1989). With respect to detoxication enzymes in the medaka, methodologies for the measurement of 7-ethoxyresorufin O-

deethylase (EROD) and Glutathione S-transferase (GST) were successfully applied. Also, existing methods and techniques for the assessment of DNA integrity (i.e., strand breaks, adduct formation, and minor nucleoside composition) were adapted.

2. Task 2.

The purpose of this task is to evaluate the effects of exposure of the Japanese medaka to known genotoxic agents on the response of a suite of molecular markers. This complex task has been subdivided into several subtasks and the progress on each is reported separately.

a. Protocols for exposure.

A protocol for exposure to DEN was reported in detail in Semi-Annual Technical Progress Report No.1 (ORNL/M-829, dated April 1, 1989). Molecular marker responses observed in Japanese medaka exposed to DEN employing this protocol have been examined (see Task 2.b. and Appendix A of technical progress report no.3).

The following three protocols for exposure to BaP were developed (see Task 2.b. below):

Exposure 1) Acclimate 60 adult Japanese medaka (Oryzias latipes) (approximately three months of age-60% female/40% male) in a 30-L aquarium at 25° for two weeks. Aerate the water continuously and renew 25% of the volume every two days. Provide food ad libitum and maintain light on a 16 hour on and 8 hour off schedule. At the end of the acclimation period, add BaP (in tween 80) to a concentration of approximately 10 ug/L (Environ. Toxicol. Chem. 8:863-869;1989). Continue regular maintenance for sixteen days, and renew 50% of volume of aquarium ever two days with water containing 10 ug/L of BaP (in tween 80). Control groups are medaka similarly treated but exposed to tween 80 (BaP vehicle) only, and medaka similarly treated, but without exposure. Remove ten fish (6 female/4 male) from each group at days zero, one, two, four, eight and sixteen. Sacrifice the animals by cervical snip and remove the following tissues: liver, gall bladder, gill, testes and ovaries. Store all tissues, including carcass, not to be used immediately in liquid N.

Exposure 2) Sixty adult medaka, acclimated to 26°C, are divided into four treatment groups with 5 fish per tank: 15 fish, untreated control; B) 15 fish exposed to verapamil (1 μg/L), an inhibitor of the P-170 glycoprotein xenobiotic pump; C) 15 fish treated with BaP (4 μg/L); and D) 15 fish treated with verapamil (1 μg/L) and BaP (4 μg/L). Maintain fish from each group in three aquaria containing 8 L of water. BaP saturated water is prepared by passage through a

generator column containing glass beads coated with BaP and the water is not renewed during a seven day exposure. Verapamil (1 µg/L) is added daily. After seven days exposure fish are sacrificed by spinal scission, the DNA extracted from whole fish and analyzed for DNA strand breaks.

Exposure 3) Eighteen adult medaka acclimated to 26°C are exposed to BaP and BaP + verapamil in a small-scale follow-up experiment. Three treatment groups are A) 6 fish, untreated controls; B) 6 fish, exposed with BaP (4 µg/L; and C) 6 fish, exposed to BaP (4 µg/L) + verapamil (1 µg/L added daily). BaP concentrations in water are maintained at saturation (4 µg/L) by continuous regeneration. After 42 h exposure, fish are sacrificed and the DNA is extracted from whole fish. DNA samples are analyzed for strand breaks, BaPDE adducts and ^{5}m-dCYD content.

A second exposure of medaka to DEN is currently underway and the results will be reported in a later progress report.

Acclimate 700 adult medaka (approximately 3 months old-60% females and 40% males) at 25°C for two weeks. There are 200 fish per tank and two tanks per treatment group and one control tank. Three exposure groups contain 100 ppm, 50 ppm, and 25 ppm. Exposure lasts for five weeks with 100% renewal of water every third day. Samples are taken at one week intervals until termination of exposure, and at 2, 4, and 8 weeks post exposure. On sampling days, ten (10) fish are taken from each tank, divided into two groups of five fish each. The livers and gills are pooled separately and carcasses kept separate. Samples are placed in 1.5 ml microfuge tubes and frozen in liquid nitrogen for shipment for DNA and protein analysis.

The following protocol was developed for exposure of medaka to AAF (2-acetylaminofluorene) and the results will be reported in a later progress report.

Medaka and King Cobra Guppy (Poecilia reticulata),(in two replicate control and AAF exposure tanks for both species; each tank contains 90 fish each; 45 males and 45 females), are exposed to 2 µg/L of 2-AAF for three days (72 h) under conditions of static exposure. Unexposed controls are included for both medaka and guppy. Fish samples treated with AAF are taken at post-exposure times 6, 12, 18, 24, 36, 48, 60, and 72 hours; controls samples though, are taken at 0, 24, 48, and 72 hours. At each sampling time, 6 males and 6 females of both medaka and guppy are taken from exposed and control tanks. The fish are weighed and individually frozen in liquid nitrogen; three specimens are pooled (males and females are separate) to give duplicate samples of each sex. After 6 h in liquid nitrogen, samples are stored at -70°C.

b. Exposure of medaka to BaP.

Medaka have been exposed to BaP according to the protocol reported in Task 2.a. above. A final, detailed report of the results of this study was made at the Annual Carcinogenicity Research Review Meeting, August 14-15 of this year, The paper submitted for this review is included in Appendix A of this report.

c. Application of new techniques & methodologies.

Flow cytometric analysis for the determination of DNA distribution in hepatocytes (Bull. Environ. Contam. Toxicol. 40:343-349;1988) has been implemented into the suite of molecular markers (see Semi-Annual Technical Progress Report No.2 for details).

Currently under consideration and evaluation are techniques to detect and measure: (a) DNA repair; (b) DNA damage due to free radicals, and cross-linking agents; (c) induction of stress proteins; (d) changes in chromatin proteins; (e) DNA associated proteins which may confer protection to the genetic material; (f) electrophoretic analysis of DNA double and single strand breaks; (g) teratogenesis study on medaka embryos; and (h) dominant lethal analysis. It is anticipated that data from these studies on the exposure of medaka to BaP, DEN, and AAF will be incorporated into future progress reports and publications.

3. Task 3.

The purpose of this task is to evaluate the short-term responses of the suite of molecular markers as suitable biomarkers to estimate exposure and predict cellular effects to genotoxic chemicals. It is anticipated that this study will provide data to demonstrate the usefulness of the Medaka as a bioassay model to evaluate the potential of environmental genotoxicity. The responses to be studied are important because they have been selected on the basis of existing experimental observations (i.e., they are key ingredients that provide evidence that exposure to genotoxic agents is resulting in toxicological interactions) and they can be used to make positive predictions.

III. PROBLEM AREAS

Because of the small size of the Medaka, and therefore the amount of tissue available for analyses, most of our procedures require adult fish for study. We currently are well stocked with adult medaka (2000) which meets our current requirements. Some planned exposures of fry are being considered because of more

rapid growth (cell division) in the young in which genotoxic effects may be more manifest. Improvements in isolation of sufficient amounts of highly purified DNA, protein, and subcellular components from medaka are being refined and should permit bioassay studies on whole fish, livers and gills. Improvements in DNA yield can permit multiple analysis (strand breaks, DNA adducts, ^{5}m-CYD etc...) from one sample. Liver and gill tissue can be easily obtained for flow cytometric analysis which can measure aneuploidy, polyploidy and other changes in nuclear DNA content. Additional molecular and cellular assays are still needed to compensate for the small size of the medaka and are being studied.

IV. WORK TO BE PERFORMED DURING THE NEXT SIX MONTHS

At present, approximately 2000 adult medaka are available for experimentation with sufficient fry being maintained to supplement those used for this purpose.

Studies will continue to focus on alterations to DNA integrity and changes in detoxication systems of medaka exposed to BaP, DEN, and AAF however, it is anticipated that exposure protocols will be modified to include mixtures of potential genotoxic agents.

A study is currently underway to determine if chromosomal proteins change as a result of xenobiotic exposure and to identify the protein factor responsible for the resistance of medaka DNA to alkaline unwinding. This resistance is induced in BaP and verapamil exposed fish. Additional studies to measure teratogenic and dominant lethal effects of xenobiotic compounds on medaka embryos have begun.

As mentioned in Task 2.c. above, the evaluation of new techniques to detect and measure DNA repair and DNA damage due to free radicals, cross-linking agents, and other DNA interacting compounds is in progress.

As data generated from the BaP exposure experiment becomes available, it will be compared to similar data generated from the DEN and AAF exposure experiments in an effort to evaluate the usefulness and effectiveness of the medaka bioassay model to predict exposure to genotoxic chemicals.

V. ADMINISTRATION COMMENTS

This report was prepared according to the instructions found in the U.S. Army Medical Bioengineering Research & Development

Laboratory document entitled "Contractor Reporting Requirements for the Health Effects Research Division" dated march 1984.

VI. GANTT CHART

Attached.

VII. COST SUMMARY REPORT

1. PROJECT COST SUMMARY

   Attached.

2. COST SUMMARY GRAPH

   Attached.

VIII. APPENDIX "A"

1. Manuscript submitted for publication in the 3rd Annual Carcinogenicity Research Review, 14-15 August, 1990, Frederick, MD. .

   Attached.

Lee R. Shugart

Lee R. Shugart, October 1, 1990

Project Title: Biological (Molecular and Cellular) Markers of Toxicity

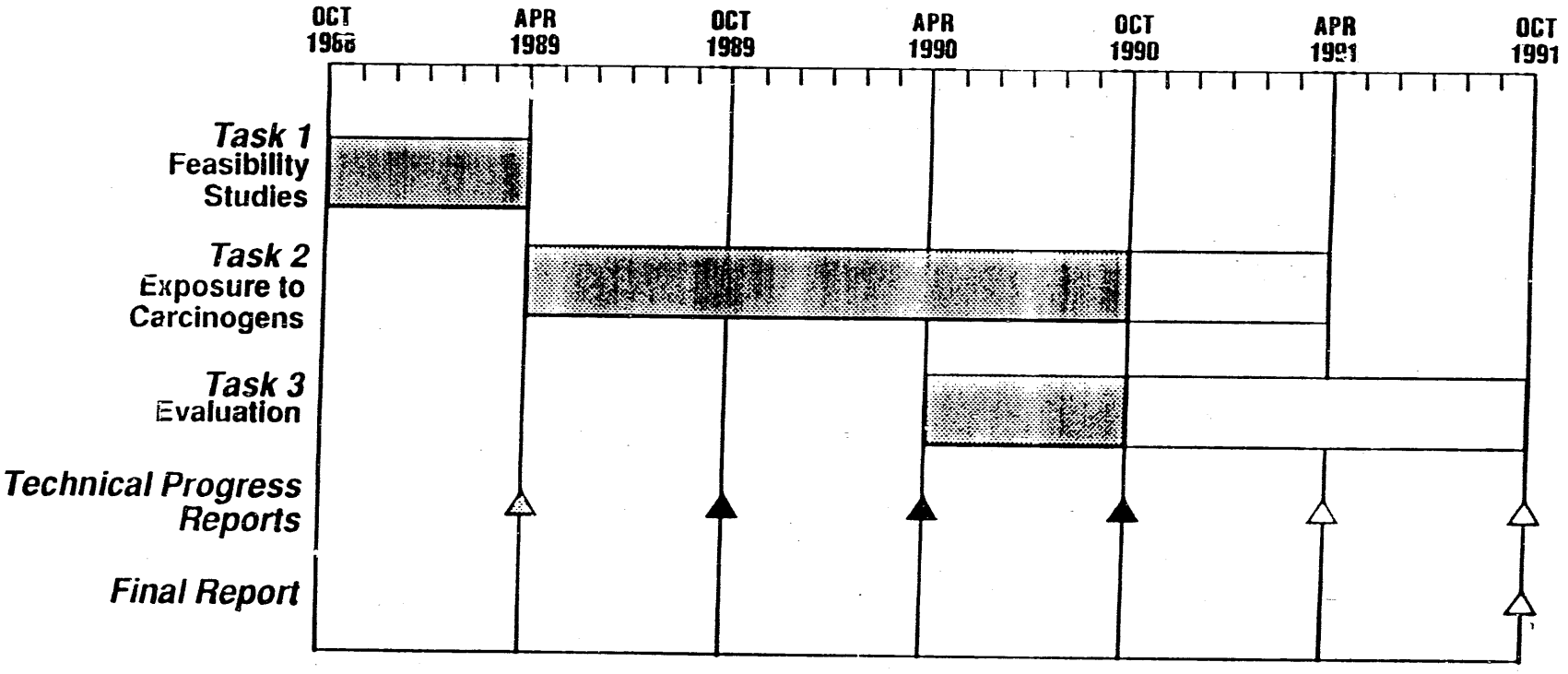
Contract No.: U.S. Army 88PP8861
IAG 1016-B047-A1

Reporting Period: October 1, 1989 to September 30, 1990

Performing Organization: Oak Ridge National Laboratory

Principal Investigator: L. R. Shugart

Date: October 1, 1990



PROJECT COST SUMMARY

Project Title No.: Biological (Molecular and Cellular) Markers of Toxicity/U.S. Army 88PP8861
Organization/PI: Oak Ridge National Laboratory/Lee R. Shugart
Reporting Period: October 1, 1989 to September 30, 1990

Total Allocation: $ 6,982 Carry-over from 1st year
$160,000 Allocated 2nd year
$166,982

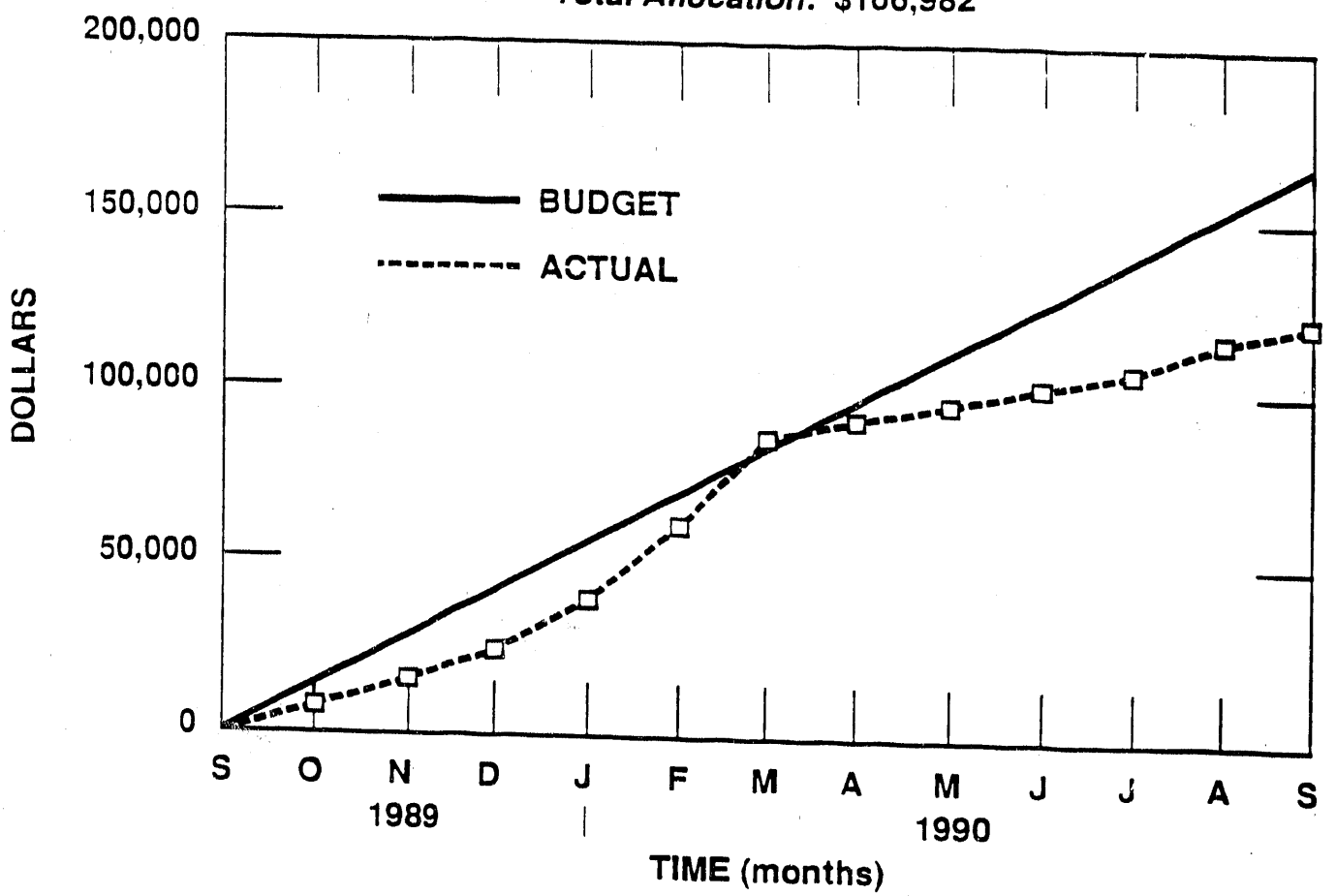
	Monthly Expenditures			Cumulative Expenditures			Available
	Budget	Actual	Variance	Budget	Actual	Variance	Balance
							166,982
Oct	13,915	7,507	-6,408	13,915	7,507	- 6,408	159,475
Nov	13,915	8,009	-5,906	27,830	15,516	-12,314	151,466
Dec	13,915	8,664	-5,251	41,745	24,180	-17,565	142,802
Jan	13,915	15,536	+1,620	55,660	39,716	-15,944	127,266
Feb	13,915	20,468	+6,553	69,575	60,184	- 9,391	106,798
Mar	13,915	26,288	+12,373	83,490	86,472	+ 2,982	80,510
Apr	13,915	5,151	-8,764	97,405	91,623	- 5,782	75,359
May	13,915	4,368	-9,547	111,320	95,991	-15,329	70,991
Jun	13,915	5,317	-8,598	125,235	101,308	-20,646	65,674
Jul	13,915	6,383	-7,532	139,150	107,691	-28,178	59,291
Aug	13,915	8,113	-5,802	153,065	115,804	-37,261	51,178
Sep	13,917	6,588	-7,329	166,982	122,392	-44,590	44,590

Note: Cost data as of end of each month.
This report was prepared 10/1/90.

ORNL-DWG 89M-9487

Cost Summary Graph

Project Title / No.: Biological (Molecular and Cellular) Markers of Toxicity / U.S. Army No. 88PP8861
Organization / PI: Oak Ridge National Lab / L. R. Shugart
Reporting Period: October 1, 1989 to September 30, 1990
Total Allocation: $166,982



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