Ref. No. [UMCEES]CBL 90-089

CONTRACT NO.: DAMD17-89-Z-9016

TITLE: IMMUNOTOXICOLOGICAL ASSAYS USING THE JAPANESE MEDAKA

PRINCIPAL INVESTIGATOR: ROBERT S. ANDERSON

PI ADDRESS: Chesapeake Biological Laboratory
University of Maryland System, CEES
P.O. Box 38
Solomons, Maryland 20688-0038

REPORT DATE: MAY 14, 1990

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK
FREDERICK, MARYLAND 21701-5012

Ref. No. [UMCEES]CBL 90-089

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Maryland; 6b. OFFICE SYMBOL (If applicable): N/A	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): P.O. Box 38, Solomons, MD 20688-0038	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-89-Z-9016
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62720A	3E1-62720A835	00	016

11. TITLE (Include Security Classification): Immunotoxicologic-1 Assays Using the Japanese Medaka

12. PERSONAL AUTHOR(S): Robert S. Anderson

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 12/15/88 TO 12/14/89	May 14, 1990	24

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (~~Continue on reverse if necessary and identify by block number~~)
FIELD	GROUP	SUB-GROUP	Immunotoxicology, bivalve mollusks, small aquarium fish, immunology, disease resistance, reactive oxygen intermediates
06	11		
06	05		~~RA-3~~

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Phagocytic blood cells secrete reactive oxygen intermediates (ROI) which are involved in immune defense reactions such as killing pathogenic microorganisms. The long-term goal of this project is to determine the effects of sublethal exposure to xenobiotics on ROI, and other immunological parameters, using a small aquarium fish model (the Japanese medaka). A relevant blood cell model system (Crassostrea virginica) was used to develop lab assays for key ROI parameters (superoxide anion, hydrogen peroxide, singlet oxygen, etc.). In addition to medaka studies, this was the first characterization of ROI in this species (oyster). The application of ROI assays to fish (goldfish, medaka, Fundulus) produced encouraging preliminary results. Exposure of C. virginica blood cells to an environmental pollutant (cadmium) produced ROI inhibition, indicative of immunosuppression. The effects of in vivo and in vitro exposure of medaka macrophages to xenobiotics on ROI are under study.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	301-663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

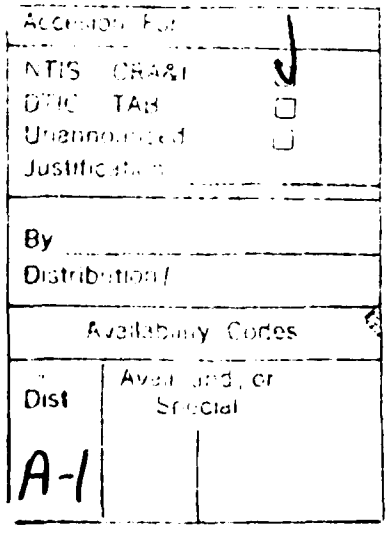
_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

RSA Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

RSA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

____In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.



Robert S. Anderson May 21, 1990
PI Signature Date

TABLE OF CONTENTS

FIGURES

INTRODUCTION

The ability of blood cells to kill pathogenic microorganisms is central to establishing resistance to infections. Modulation of these capacities resulting from chemical exposure would have serious consequences to the health and survival of individuals and/or populations. Therefore, we chose to focus on blood cell-mediated antimicrobial mechanisms in our studies of immunotoxicity. Specifically, we have been studying the production of cytotoxic oxygen radicals by blood cells (hemocytes) withdrawn from the oyster (Crassostrea virginica). Although the ultimate goal is to quantify the generation of reactive oxygen metabolities by medaka blood cells, the oyster hemocyte model was selected for several reasons. 1) the first year of this grant was devoted to adaptation of methodology originally developed for immunological evaluation of mammalian and human leukocytes for use with aquatic species. This adaptation and optimization required large quantities of blood cells which would have depleted the available medaka supply. 2) We routinely hold large numbers of oysters in our flow-through aquaria; each oyster can yield 3-10 ml of hemolymph, containing $1\text{-}2X10^6$ cells/ml. 3) Oyster hemocytes are morphologically and functionally similar to macrophages, a common phagocytic cell type widely distributed in animals at most phyletic levels (including fish).

The specific assays developed in this lab to measure blood cell antimicrobial capacity (immunological competency) include: 1) chemiluminescent responses of hemocytes, 2) superoxide anion production, and 3) hydrogen peroxide (H_2O_2) production. Hemocytes respond to specific

membrane stimuli and phagocytosis by the production and extracellular release of reactive oxygen reduction products. This coordinated sequence of reactions is initiated by increased oxygen uptake followed by the one-electron reduction of oxygen to superoxide anion (O_2^-), which is subsequently converted to H_2O_2 spontaneously or by the enzyme superoxide dismutase. Both O_2^- and H_2O_2 have been shown to be specifically involved in cytotoxic mechanisms directed against both microorganisms and tumor cells. Associated with the production of active oxygen species is the generation of photons by the hemocytes; therefore, chemiluminescence is frequently used to quantify killing activities of the cells. NBT reduction is also viewed as a measure of the oxidative events associated with cellular killing and has been used clinically to identify defects in leukocyte defense mechanisms. NBT reduction has classically been evaluated under the microscope by observing the characteristic intracellular depositions of blue-black reaction products; however, we use a more precise spectrophotometric extraction method. In addition to NBT reduction as a measure of intracellular O_2^- production, we measure the release of O_2^- and H_2O_2 by oyster hemocytes by ferricytochrome c reduction and phenol red oxidation, respectively.

The above methods offer many advantages over more traditional ways to estimate cellular killing potential, particularly as related to their rapidity, sensitivity, quantitative nature, lack of subjectivity, and potential for gathering accurate data from large (highly statistically-significant) numbers of hemocytes.

BODY

IMMUNOASSAYS DEVELOPED

A major portion of the first year of the grant was devoted to developing assays to detect the production of reactive oxygen intermediates (ROI) by blood cells of aquatic animals. The following assays have been shown in our lab to provide precise, quantitative data on ROI produced by the blood cells (macrophages) of oysters and several species of fish. These methods quantify the potential of cells to destroy many kinds of pathogenic microorganisms, thereby indicating the immune status of the host and providing an exact means to quantify chemically-induced immunomodulation.

Quantitative NBT Reduction. The original protocols for nitroblue tetrazolium reduction assays involved visual evaluation of the reaction in blood cell monolayers on slides. NBT is a pale yellow dye that is reduced by superoxide to a dark-blue, water insoluble product called formazan. Normal phagocytes show markedly augmented formazan production during phagocytosis or when their membranes are appropriately stimulated. We have observed stimulated NBT reduction on slide preparations of oyster blood cells and also have developed a quantitative, spectrophotometric assay for NBT reduction by hemocytes.

Hemocytes are phagocytically stimulated by intact yeast or zymosan particles (a preparation of yeast cell wall components) for 30-60 min in the presence of NBT, and the reaction is stopped by the addition of 0.5N HCl. Centrifuged supernatants are extracted 2x with pyridine at 100°C, the extracts are pooled for each sample and the delta OD at 515 nm/30-60 min/mg protein is calculated. Unstimulated (resting) NBT reduction values are also obtained in the absence of phagocytic stimuli.

Interpretation of data from these studies provides information on the ability of the phagocytes to respond to immunological stimulation (the increment in NBT reduction produced by phagocytosis of zymosan), as well as on the basal level of antimicrobial ability of the blood cells (a reflection of natural background levels with influences from present infection patterns superimposed). Both resting and stimulated NBT reduction levels can be specifically modulated by exposure of the host organism to environmental chemicals (Fig. 1).

Superoxide anion. Release of O_2^- from phagocytes is measured by the reduction of ferricytochrome c (Type III, Sigma). Hemocyte monolayers in 3 cm plastic tissue culture plates are bathed in 1 ml of 80 µM ferricytochrome c. The cells are stimulated with zymosan particles or phorbol myristate acetate (PMA) and the preparations are incubated 90 min, at 30°C, in a 95% air - 5% CO_2 atmosphere. Cytochrome c reduction by unstimulated cells is also quantified. After incubation, the supernatants are removed, centrifuged free of any cells or particles (5 min, 2000 xg, 4°C), and the optical density is measured at 550 nm against blanks of cytochrome c solution incubated without hemocytes. The concentration of cytochrome c reduced is calculated: delta E_{550} nm = 1.2 X $10^4 M^{-1} cm^{-1}$ (Fig. 2). The specificity of cytochrome reduction by O_2^- is checked by the introduction of 300U/ml superoxide dismutase (SOD) during incubation with cytochrome c.

This method complements the NBT reduction technique, which quantifies intracellular production of O_2^- by measuring the amount of superoxide anion released into the extracellular medium. In addition, this method maybe particularly useful for medaka studies, where the cell numbers are limited,

because it can be adapted for use in microtiter plate wells, in which reactions can be run in <300 µm volumes and the resultant color reactions read with a standard ELISA spectrophotometer.

H_2O_2 Release. A simple method to quantify H_2O_2 by phagocytic cells was adapted from a clinical hematology function assay, which is also amenable to ELISA technology. It is based on the H_2O_2-mediated and horseradish peroxidase (HRPO)-dependent oxidation of phenol red to a product with an absorption maximum of 610 nm. The hemocytes are established in 3-cm culture dishes and covered with 5.5 mM dextrose, 10 mM phosphate buffer (pH 7.0), 0.28 mM phenol red and 8.5U/ml of HRPO. The usual stimulants (zymosan or PMA) are added; controls contain either no stimulants or no hemocytes. After incubation, cell-free supernatants are removed, centrifuged (5 min, 2000 xg, 4°C) and made alkaline by the addition of 10 µl of 1N NaOH, and the OD is read at 610 nm against a blank of phenol red solution treated with 1N NaOH (Fig. 3). H_2O_2 is expressed at nM H_2O_2/mg cell protein, using standard OD curves generated from H_2O_2 standards (Fig. 4).

Chemiluminescence. In this method approximately $2X10^6$ hemocytes are incubated without (unstimulated sample) or with (stimulated) heat-killed yeast cells, zymosan, or phorbol myristate acetate (PMA) and luminol. The resultant cellular chemiluminescence is quantified in a Packard liquid scintillation counter programmed for single photon counting. Luminol is included in the medium to amplify the chemiluminescent signal and the data are recorded every 12 sec for 30-45 min. The data are plotted, peak values and times are recorded, as well as the areas under the curves and the initial kinetics of the response. Chemiluminescence correlates well with bactericidal events (such as singlet oxygen and/or O_2 production) associated with hemocyte

stimulation. The intensity of this activity can be precisely measured and preliminary evidence indicates that it is subject to modulation by xenobiotic exposure.

Peak responses of >1M cpm per 10^6 cells are routinely generated after PMA stimulation in the presence of luminol. The response is SOD inhibitable and responds predictably to other control conditions, cell densities, and is stimulant dose-dependent, etc.

SUMMARY OF RESULTS WITH OYSTER HEMOCYTES

An early indication of ROI production by oyster hemocytes was seen microscopically in monolayer primary cultures by the reduction of nitroblue tetrazolium (NBT). NBT reduction is accepted as a manifestation of superoxide (O_2) production, which was verified in our preparations by inhibition by superoxide dismutase (SOD). Rather than depend on visually assessing the relative amount of NBT formazan present in hemocytes from animals in different treatment groups, the actual concentration of reaction product was measured spectrophotometrically before and after phagocytic stimulation. The extraction efficiency of the procedure is very high and a relatively strong response was demonstrated in both stimulated and unstimulated cells. To optimize conditions we determined that the NBT concentration was not cytotoxic (by trypan blue exclusion), the optimal yeast:hemocyte ratio, the optimal time for hemocyte-particle interaction, and the kinetics of the reaction. These factors are all incorporated in the final protocol (Fig. 1). For oysters (Crassostrea virginica), NBT reduction in unstimulated blood cells ranged from about 0.04 to 0.15 $OD_{515}/2X10^6$ cells/hr; phagocytic stimulation produced an increment of about 70% in this activity. These data compare favorably to

those from other species.

Another method used to measure O_2 generation by blood cells involved the reduction of cytochrome c present in the culture medium. Rather than use cells in suspension as in the NBT reduction assay, we used surface-adherent blood cells, this provides excellent cell homogeneity because of the highly adherent property of macrophages. The assay was optimized, as previously described, and found to be stimulant dose-dependent and SOD-sensitive. A stronger signal was generated with the membrane stimulator phorbol myristate acetate (PMA) than phagocytic stimulators such as heat-killed yeast or zymosan. The reaction took place rapidly (within 5 min.), but seemed to be inhibited by the continued presence of PMA in the culture medium. Therefore, we stimulated the cells by pretreatment with PMA, followed by a PMA-free medium wash, prior to overlaying the cells with ferricytochrome c. This is summarized in fig. 2. Generally, the mean OD_{550} red, cytochrome c/2X10^6 cells/hr. was about 0.05 to 0.10; preincubation with PMA produced about a 10-50% increase in activity. However, the results of this assay showed considerable individual variation between oysters and sometimes produced no detectable cytochrome c reduction. We find this assay of O_2 activity to be less useful than NBT reduction for potential immunotoxicological application.

H_2O_2 production by C. virginica hemocytes, as measured by phenol red oxidation, seemed to provide a sensitive and reproducible indicator of ROI. The assay, like the cytochrome c assay for O_2, employs a monolayer of adherent blood cells which can be stimulated by PMA preexposure. The conditions were optimized for this assay, which is summarized in Fig. 3. Parameters checked included cell viability at all stages, adjustment of cell numbers to assure that resultant absorbances fell on the linear portion of the standard curve,

determination of optimal PMA concentration and duration of preincubation period, and effect of cell density on H_2O_2 production. The assay has merit as a screening method to identify potential immunotoxicants. Fig. 5 shows the effect on H_2O_2 production of in vitro exposure of hemocytes to Cd. There is a dose-dependent inhibition of this important protective, bactericidal mechanism occurring at sublethal exposure levels of the metal. While clearly the Cd concentration needed to produce this inhibition is minimally cytotoxic, it is higher than levels probably encountered in vivo; however, this effect was measured simultaneously with exposure. The sensitivity of this response can probably be greatly enhanced by looking for metal-induced immunosuppression in hemocytes cultured for longer periods in the presence of markedly reduced Cd levels. These studies are underway. These preliminary results provide evidence of a mechanism (impaired ROI production) that may be the basis for immunotoxicity in C. virginica produced by exposure to an environmental xenobiotic.

Macrophage chemiluminescence (CL) can be directly correlated with cellular ability to kill pathogenic bacteria and other microorganisms. We have programmed our liquid scintillation counter to measure this phenomenon and have found this to be the most sensitive and straightforward way to measure blood phagocyte ROI. To perform the assay $1-2X10^6$ blood cells are mixed with a suspension of heat-killed yeast particles and luminol in dark-adapted scintillation vials. An identical aliquot of cells and luminol, but no phagocytic stimuli, are run simultaneously to determine background CL. The magnitude of the CL response in the presence of stimulant is impressive, often >1-2M cpm. More importantly for this study of immunotoxicology, the induced response (a measure of the cidal capacity of the blood cells) can be virtually

eliminated by in vitro exposure of the hemocytes to Cd (Fig. 6).

EXPERIENCE WITH MEDAKA HUSBANDRY

Sources and Maintenance in the Laboratory

An entire wet lab module has been dedicated to the support of a medaka colony for this project on immunotoxicology. Our first experience with medaka (obtained from the colony at Ft. Detrick) was disappointing, pointing up the deficiencies of our well water. These medaka soon became sick and died; however, we have subsequently addressed many of our early problems with husbandry. This has taken many months and many medaka. Rather than recount these efforts in detail, we will summarize our present methods for maintaining these small aquarium fish.

1. Sources of medaka:

Underwater Exotics 175-35 148th Rd. Jamaica, NY 11434	medaka imported from Japan, must pick up at airport
Aquaria West, Inc. 6406 Baltimore National Pike Catonsville, MD 21228	medaka imported from Japan fish are held at Aquaria West until needed. Can supply in quantity, 2 wks. notice required

2. Water: deep well water extensively aerated with air stones
   pH adjusted to about 7 with Negative pH Buffer (Marine Enterprises, Inc.) or pH Down (Aquarium Pharmaceuticals)
   Water Softener Pillow (Aquarium Pharmaceuticals) placed in filters
   Filtration: Whisper Power Filter 1 (Second Nature) with BioBag disposable filter cartridges
   Heater: Supreme Heatmaster - back-up on each tank in case room heat fluctuates
   Bacterial supplement: Cycle

3. Food for fry: Interpet Liquifry No. 1 for Egglayers and TetraMin Baby Fish Food "E" for Egglayers, baby brine shrimp (frozen).

4. Food for adults: TetraMin flake food supplemented with Kordon Polar Seas Freeze-dried Ocean Plankton and live brine shrimp 3X/wk.

5. Treatment for infectious diseases: Malachite Plus (Marine Enterprises, Inc.)

Currently, we have 12 ten-gallon tanks for housing adults (~40/tank) plus numerous smaller tanks for fry at different life stages.

Using the above procedures, we have no problem maintaining medaka in apparently good health with very little mortality. After several months the condition of our first shipment of fish declined and the mortality rate increased. The explanation for this was unknown, perhaps the fish had some infectious disease (parasites?) that took some period of time to produce pathological effects. Malachite green treatment had little positive effect. However, we successfully bred the adults while they were still in excellent condition by adjusting the temperature 26°C and the photoperiod to 16L:8D. Eggs were transferred daily to petri plates containing embryo rearing medium (Carolina Biological Supply Co.). After hatching, the fry were transferred to 2.5 gallon aquaria and fed fry food as above. Very few of the hundreds of fry raised so far show any signs of disease and the mortality rate is minute. The fry have reached reproductive age and they seem to be completely healthy and are expected to start breeding soon. At this time we anticipate maintaining our own breeding colony; allowing total independence from commercial suppliers and from diseases introduced with new shipments of fish.

CURRENT RESEARCH ACTIVITIES, PLANS FOR YEAR TWO

1. Phase out of oyster hemocyte work. Complete already started studies of effects of *in vitro* exposure of hemocytes to Cd and other environmental xenobiotics with regard to their production of cytotoxic oxygen radicals. Finish writing and submit papers on this work (manuscripts listed below).

2. We now have a suite of immunoassays completely ready to use on the medaka project, including the state-of-the-art reactive oxygen metabolite tests. The major technical problem is the paucity of phagocytic cells available from this small fish. We are approaching this difficulty vigorously and are comparing the yield of cells using several methods 1) bleeding from caudal vein, 2) tissue disruption/single cell preparation from spleen and/or head kidney, and 3) obtaining peritoneal macrophages by lavage before and after elicitation with various agents. We are hopeful that the use of one or more of these techniques will enable us to apply our methods to study various aspects of cell-mediated immunity in the medaka. In addition, we plan to study the feasibility of measuring immunoglobulin profiles and the activities of other immunomolecules in medaka plasma samples.

3. Preliminary studies of ROI in goldfish and Fundulus indicate that these larger species provide abundant macrophagesfor immunotoxicological studies. The best source of macrophages for these studies is found in the anterior (head) kidney; this organ is comparatively large and can be easily removed from either goldfish or Fundulus, but is very small in medaka. We have already generated considerable preliminary data on ROI in individual goldfish and Fundulus, head kidneys must be pooled from 6-8 medaka to yield enough tissue. Traditional techniques to obtain peritoneal macrophages from medaka have yielded poor results, as have attempts to obtain peripheral blood. These limitations do not hold for Fundulus, or goldfish. Therefore, it is recommended that Fundulus be considered as an alternative to the medaka as a model for immunotoxicity testing. Besides its considerable advantages from a technical point of view with regard to the immunoassays, Fundulus is a small fish commonly used in many kinds of experimentation, it is a locally abundant

species, it is readily maintained in the laboratory, and has ecological significance in the Chesapeake Bay and elsewhere on the East Coast.

PAPERS IN PREPARATION (as a result of year one support)

1. NBT reduction patterns in adherent hemocytes of Crassostrea virginica.
2. Quantitative assays of superoxide anion and hydrogen peroxide production by oyster blood cells.
3. Effects of cadmium exposure on oxygen radical generation by oyster hemocytes.

SYMPOSIA

The following symposia were organized and chaired by R.S. Anderson and dealt entirely or in part with molluscan or fish immunotoxicology.

1. Symposium on "Sublethal Indicators of Toxic Stress; Laboratory Techniques and Field Validation." ASTM 13th Symposium on Aquatic Toxicology and Risk Assessment. Atlanta, GA, April 1989.
2. Symposium on "Molluscan Pathobiology." Annual Meeting of the Society for Invertebrate Pathology. College Park, MD, August 1989.

Robert S. Anderson was invited to present a paper at the following symposium; it contains data generated under this grant.

1. Symposium on "Effects of Anthropogenic Inputs on Bivalves." 82nd Annual Meeting of the National Shellfisheries Association. Williamsburg, VA, April 1990.

CONCLUSIONS

1. The ability of phagocytic blood cells to destroy bacteria and other microorganisms is central to establishing and maintaining resistance to infectious diseases and parasitism. This killing capacity depends on the blood cells' capacity to produce various cytotoxic reactive oxygen intermediates (ROI). The major goal of this study is to quantify the effects on blood cell-generated ROI of exposure of a small aquarium fish (the Japanese Medaka) to sublethal concentrations of environmental chemicals. Depression in blood cell-generated ROI is indicative of immunosuppression and is considered to have profound effects on the health and survival of affected individuals or populations.

2. The following ROI immunoassays were developed during year one of this grant for use with the medaka. An oyster hemocyte model was used for these preliminary studies because of the relative paucity of blood cells (macrophages) available from medaka and because of the great physiological similarity between macrophages at all phyletic levels. Superoxide (O_2^-) production was quantified by nitroblue tetrazolium (NBT) reduction or by ferricytochrome c reduction. NBT reduction seemed to provide the best measure of O_2^- production and/or release. Hydrogen peroxide generation was assayed by a phenol red oxidation method. Chemiluminescent (CL) responses of macrophages were used to measure production of ROI such as O_2^- and singlet oxygen. The methods proved to be quite sensitive and can be adapted for use with very small blood cell numbers ($0.5\text{-}1.0X10^6$).

3. Preliminary studies using fish that have larger lymphoid organs (Fundulus and goldfish) showed that the information gained from the C.

virginica (oyster) blood cell model system was relevant to the fish system. Both head kidney and splenic macrophages were obtained by mild organ disruption and were purified by discontinuous density gradient separation. Macrophages from both anatomical sources in goldfish and in Fundulus and from head kidney in medaka produced strong CL responses, particularly after phagocytic stimulation. We have no reason to doubt that the other ROI parameters under study can also be demonstrated with fish macrophages. Preliminary studies suggest that by pooling tissue from 6-8 medaka enough head kidney macrophages can be obtained to run the ROI tests as part of an immunotoxicological assessment evaluation.

4. In vitro exposure of C. virginica macrophages to cadmium, a common metal aquatic pollutant, was shown to be strongly immunosuppressive (at otherwise non-cytotoxic concentrations) by several of the ROI assays. Both H_2O_2 production and chemiluminescence were markedly inhibited. These results encourage further in vivo studies of immunotoxicology via ROI methodology in both bivalves and fish.

QUANTITATIVE NBT REDUCTION ASSAY

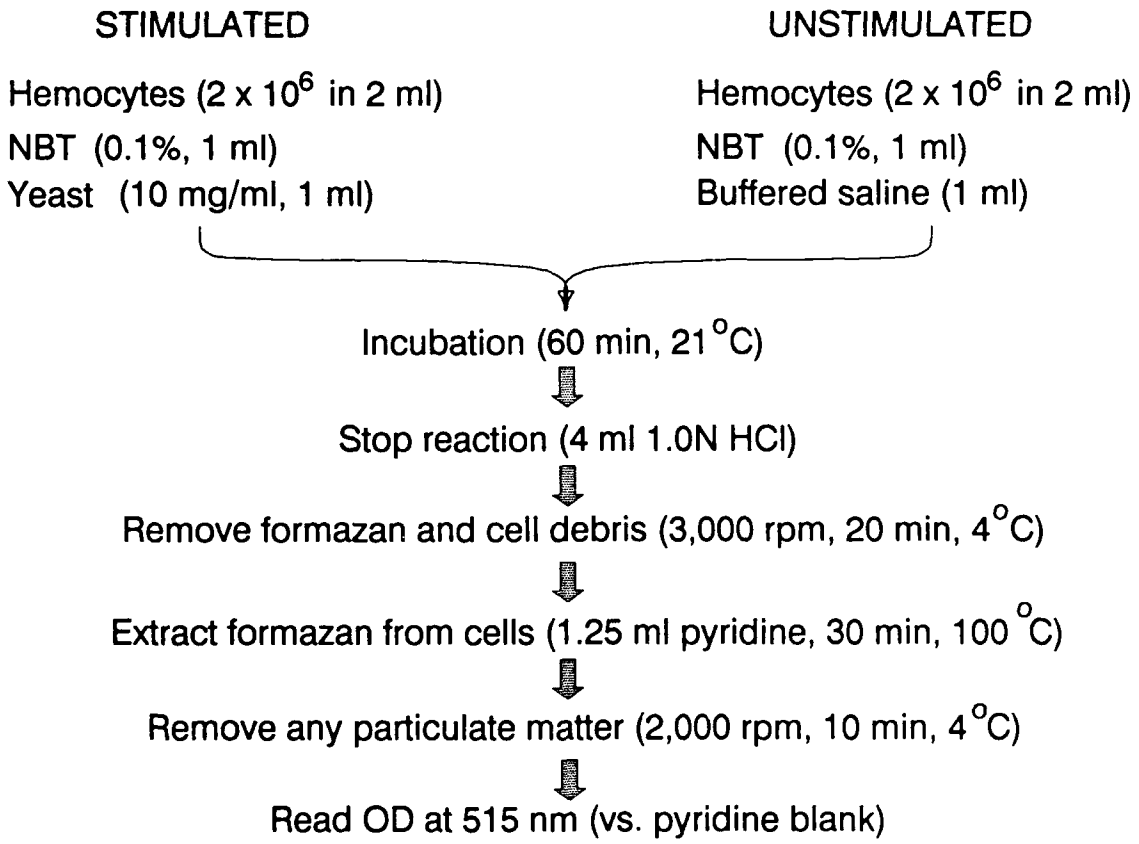


Figure 1. Quantitative NBT Reduction Assay.

SUPEROXIDE GENERATION ASSAY

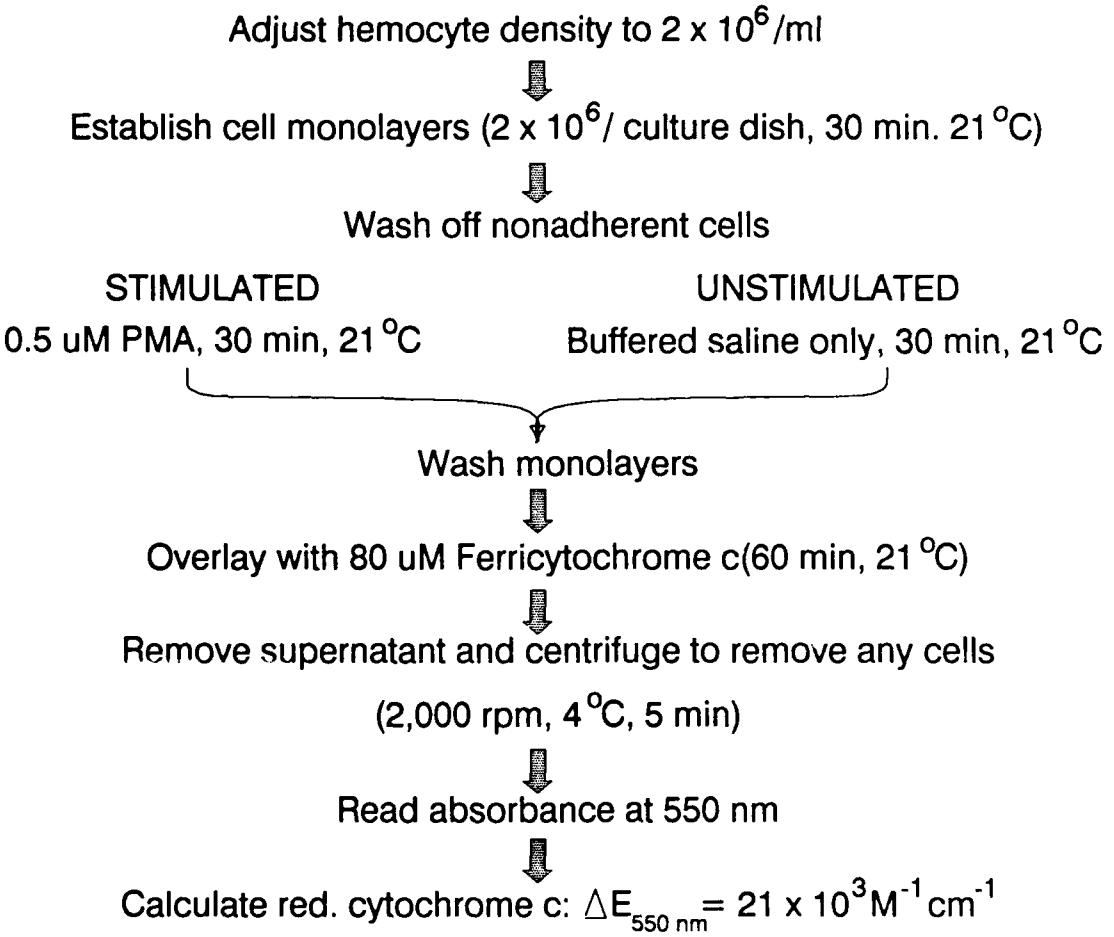


Figure 2. Superoxide Anion Generation Assay.

H_2O_2 GENERATION ASSAY

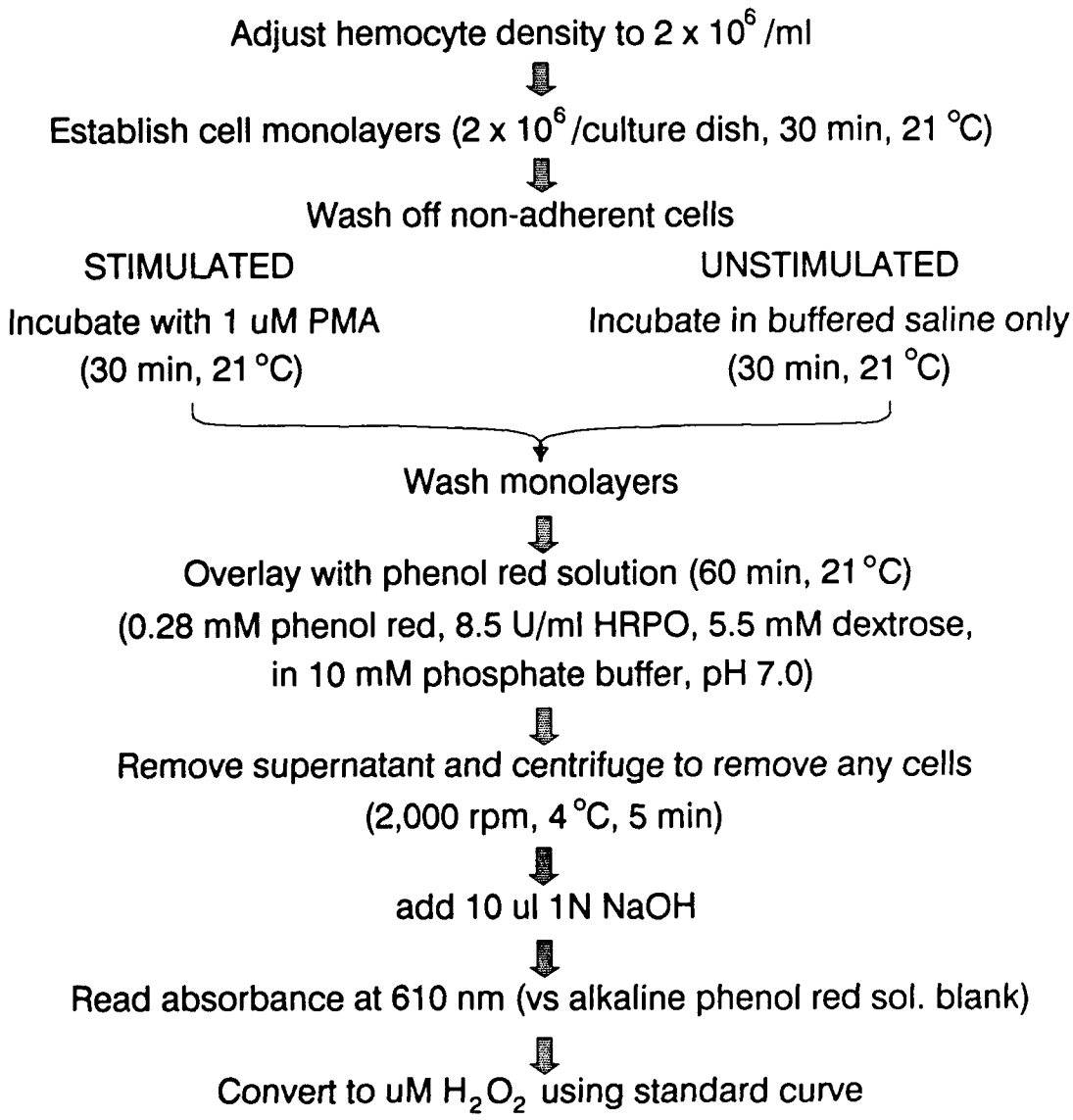


Figure 3. Hydrogen Peroxide Generation Assay.

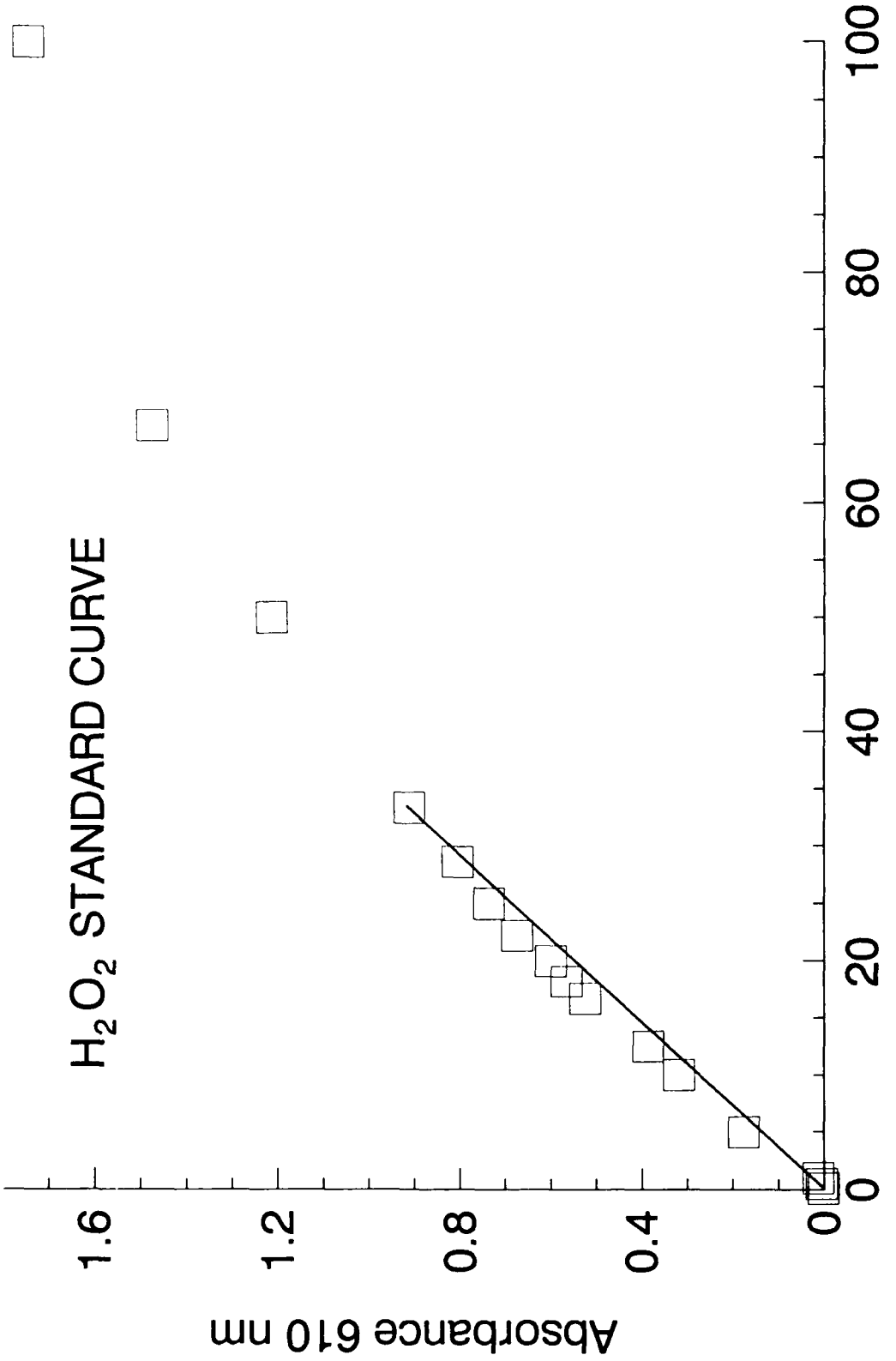


Figure 4. Hydrogen Peroxide Standard Curve.

EFFECT OF IN VITRO EXPOSURE OF OYSTER HEMOCYTES TO CADMIUM (60 MIN EXPOSURE) ON H_2O_2 PRODUCTION

Cd CONCENTRATION	ΔOD_{610nm} PMA STIMULATION	CELL VIABILITY TRYPAN BLUE EXCLUSION
0	115	2.6
2.5 ppm	110	2.5
20	102	3.2
40	104	3.0
50	115	2.9
100	122	2.1
200	95	2.9
400	106	2.7
500	88	2.3
2.5 ppt	12	5.3
5	0	7.2

Figure 5. Effect of Cadmium on H_2O_2 Generation by C. virginica Hemocytes.

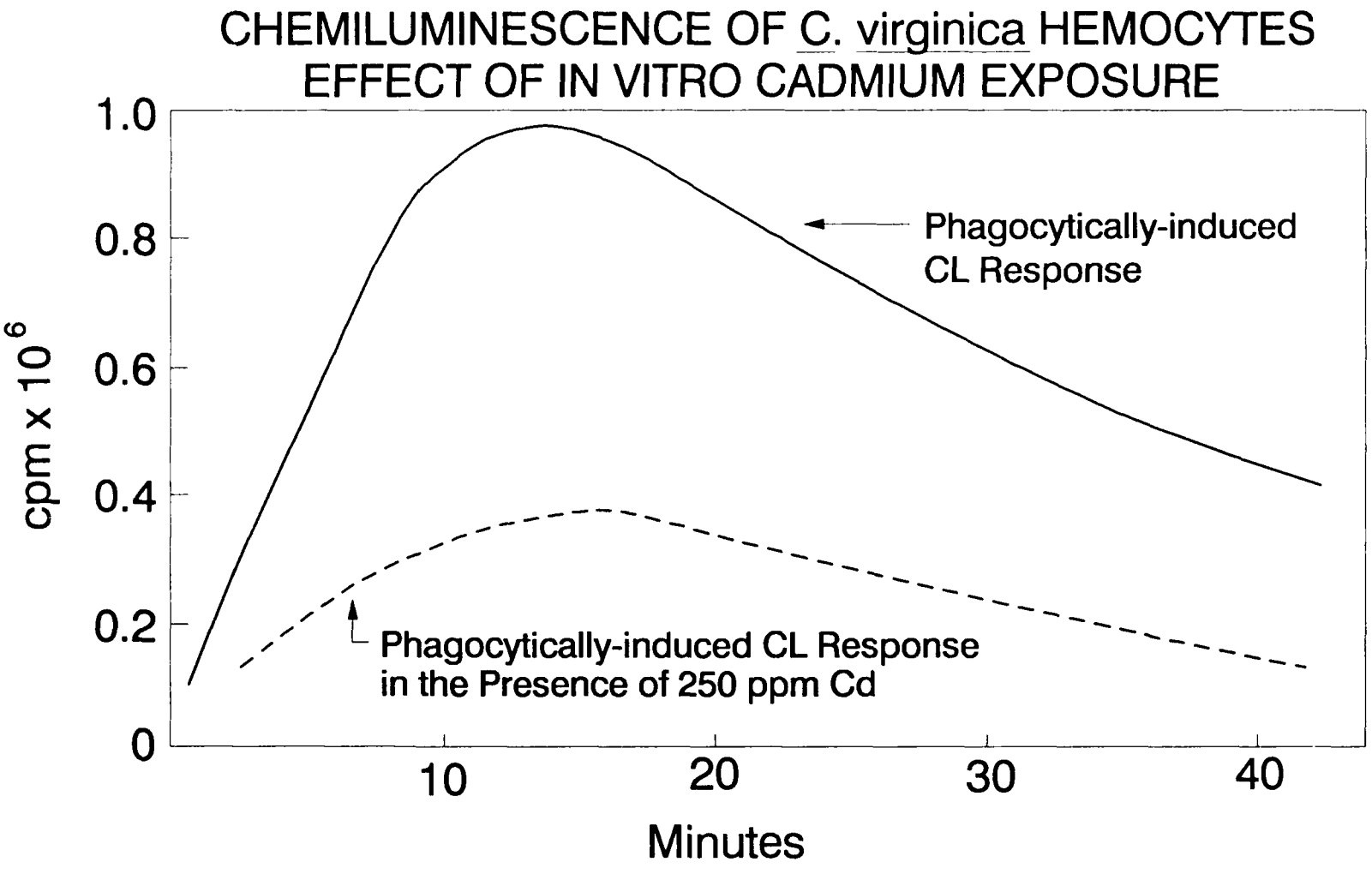


Figure 6. Effect of Cadmium on Chemiluminescent Response of C. virginica Hemocytes.