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Oak Ridge
Associated
Universities

October 26, 1981

Dr. William R. Bibb, Director
Research Division
Department of Energy
Oak Ridge, TN 37830

Subject: GRANT APPLICATION TO NIH FOR A PROJECT ENTITLED *LIPID METABOLISM AND DIFFERENTIATORS OF LEUKEMIA*

Dear Dr. Bibb:

Enclosed are three copies of the subject grant application to NIH. The proposed project will be carried out under the direction of Dr. Myles Cabot. Draft copies of this application were forwarded to your office for review on September 18, and approval for formal submission was given by Dr. Richard Benson on September 29.

Should this project be approved by NIH, the work will be carried out under policies and procedures previously established between ORAU and DOE.

We will keep you advised of NIH action on this proposal.

Sincerely,

William E. Felling

William E. Felling
Acting Executive Director

RYAN:br

Enclosures

REPOSITORY Oak Ridge Operations
COLLECTION Records Holding area Documentation 1944-94
BOX No. B-87-9 Bldg. 2714-H
FOLDER 20-88-81 NIH

20-88-81

1079537

Oak Ridge
Associated
Universities

Post Office Box 117
Oak Ridge, Tennessee 37830
Telephone 615 576-3310

Executive
Office

October 26, 1981

Division of Research Grants
National Institutes of Health
U. S. Public Health Service
Bethesda, Maryland 20205

Gentlemen:

We are submitting for your consideration seven copies of a grant application entitled *Membrane Lipids and Differentiators of Leukemia*. This project will be supervised by Dr. Myles Cabot.

Oak Ridge Associated Universities is a nonprofit corporation sponsored by 51 Southern colleges and universities. The major portion of its activities are carried out under a long-term operating contract with the U. S. Department of Energy. Certain conditions arising from this relationship between ORAU and the DOE are set forth on pages 10 and 11 of this application.

If questions should arise during the review of this proposal, please do not hesitate to call Dr. Cabot at area code 615, 576-3122.

Sincerely,

Original Signed By
WILLIAM E. FELLING

William E. Felling
Acting Executive Director

br

Enclosures

bcc: Dr. William R. Bibb DOE ORO (3)
Executive Office (2)
W. F. Countiss
M. Cabot
B. P. Ryan

FORM APPROVED
O.M.B. NO. 68-R0249

DEPARTMENT OF HEALTH, ED TION, AND WELFARE
PUBLIC HEALTH SERVICE

GRANT APPLICATION

FOLLOW INSTRUCTIONS CAREFULLY

LEAVE BLANK		
TYPE	ACTIVITY	NUMBER
REVIEW GROUP		FORMERLY
COUNCIL/BOARD (Month, year)		DATE RECEIVED

1. TITLE OF APPLICATION (Do not exceed 56 typewriter spaces)
MEMBRANE LIPIDS AND DIFFERENTIATORS OF LEUKEMIA

2. RESPONSE TO SPECIFIC PROGRAM ANNOUNCEMENT [X] NO [ ] YES (If "YES," state RFA number and/or announcement title)

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (Last, first, middle)
CABOT, MYLES C.

3b. SOCIAL SECURITY NUMBER
[redacted]

3c. MAILING ADDRESS (Street, city, state, zip code)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

3d. POSITION TITLE
Biochemist

3e. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
Biological Chemistry

3f. TELEPHONE (Area code, number and extension)
615-576-3122

3g. MAJOR SUBDIVISION
Medical and Health Sciences Division

4. HUMAN SUBJECTS, DERIVED MATERIALS OR DATA INVOLVED
[XX] NO [ ] YES (If "YES," form HEW 596 required)

5. RECOMBINANT DNA RESEARCH SUBJECT TO NIH GUIDELINES
[XX] NO [ ] YES

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)
From: July 1, 1982 Through: June 30, 1987

7. TOTAL DIRECT COSTS REQUESTED FOR PROJECT PERIOD (from page 5)
$413,900
$

8. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD (from page 4)
$ 71,000

9. PERFORMANCE SITES (Organizations and addresses)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P.O. Box 117
Oak Ridge, Tennessee 37830

10. INVENTIONS (Competing continuation application only)
Were any inventions conceived or reduced to practice during the course of the project?
[X] NO [ ] YES - Previously reported
[ ] YES - Not previously reported

11. APPLICANT ORGANIZATION (Name, address, and congressional district)
Oak Ridge Associated Universities
P.O. Box 117
Oak Ridge, Tennessee 37830
Third Congressional District

12. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT (See instructions)
Code 60 Description:
(Medical and Health Sciences Division)

13. ENTITY IDENTIFICATION NUMBER
162047681A1

14. TYPE OF ORGANIZATION (See instructions)
[XX] Private Nonprofit
[ ] Public (Specify Federal, State, Local):

15. OFFICIAL IN BUSINESS OFFICE TO BE NOTIFIED IF AN AWARD IS MADE (Name, title, address and telephone number.)
William F. Countiss
Head, Office of Fiscal Services
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830 (515 576-3056)

16. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Name, title, address and telephone number)
William E. Felling
Acting Executive Director
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830 (615, 576-3300)

17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a (In ink. "Per" signature not acceptable)
[signature]
DATE
OCT 22, 1981

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense. (U.S. Code, Title 18, Section 1001.)

SIGNATURE OF PERSON NAMED IN 16 (In ink. "Per" signature not acceptable)
William E. Felling
DATE
10/27/81

PHS-398

0109539

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

ABSTRACT OF RESEARCH PLAN

LEAVE BLANK

PROJECT NUMBER

NAME AND ADDRESS OF APPLICANT ORGANIZATION *(Same as Item 11, page 1)*
Oak Ridge Associated Universities, P. O. Box 117
Oak Ridge, Tennessee 37830

TITLE OF APPLICATION *(Same as Item 1, page 1)*
MEMBRANE LIPIDS AND DIFFERENTIATORS OF LEUKEMIA

Name, Title and Department of all professional personnel engaged on project, beginning with Principal Investigator/Program Director

Myles C. Cabot, Ph.D., Biochemist, Biological Chemistry, Medical and Health Sciences Division

ABSTRACT OF RESEARCH PLAN: Concisely describe the application's specific aims, methodology and long-term objectives, making reference to the scientific disciplines involved and the health-relatedness of the project. The abstract should be self-contained so that it can serve as a succinct and accurate description of the application when separated from it. DO NOT EXCEED THE SPACE PROVIDED.

The tumor promoter, 12-O-tetradecanoylphorbol-13-acetate, and compounds such as dimethyl sulfoxide and retinoic acid, induce differentiation of cultured human leukemia cells. Because these chemicals could function as potential chemotherapeutic agents, knowledge of the mechanisms by which they act is of critical importance. Profound alterations of lipid metabolism have been demonstrated in induced cells, most notably the stimulation of phospholipid metabolism before the markers of differentiation are expressed. The inducers are lipophilic in nature and cell membranes are strong targets of action. The parallel between membrane action and lipid modulation strongly suggests that lipids play a role in the cellular differentiation process. Because phorbol esters and other inducers display such a varied spectrum of lipid-modifying activities, the necessity of a systematic study of lipid metabolism in induced cells is apparent, with potential to produce insight into the role of membrane lipids in cellular differentiation. The experimental approach in this proposal entails use of agents that potentiate leukemic cell differentiation in order to formulate a corrolary between the induction process and biomembrane lipid modifications. A basic question being: are similar changes in lipid metabolism common to differentiation without regard to the agent of induction? Secondly, by using human leukemia cells that do not undergo differentiation when exposed to phorbol esters, we can determine the effects of inducer on lipid metabolism, per se. The proposed investigations will be done with cultured human leukemia cells treated with either 12-O-tetradecanoylphorbol-13acetate, dimethyl sulfoxide, retinoic acid, actinomycin-D, hexamethylene-bis-acetamide, or hypoxanthine. The lipid compositions as well as in vitro enzymatic activities of key lipid metabolic enzymes will be examined in control, inducer resistant, and differentiated cell populations. Subcellular fraction targets and lipid requiements (essential fatty acids, prostaglandins) of cellular differentiation will be critically assessed.

LABORATORY ANIMALS INVOLVED. Identify by common names. If none, state "none"

None

TABLE OF CONTENTS

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 5a, 5b. Type the name of the Principal Investigator/Program Director at the top of each printed page and each continuation page.

SECTION 1.

SECTION 2.

SECTION 3. Appendix *(Six sets) (No page numbering necessary for Appendix)*

Number of publications: 1 Number of manuscripts: 1

Other items (list):

One curriculum vitae

Application Receipt Record, form PHS 3830
Form HEW 596 if Item 4, page 1, is checked "YES"

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: Myles C. Cabot, Ph.D.

DETAILED BUDGET FOR FIRST 12 MONTH BUDGET PERIOD
DIRECT COSTS ONLY

FROM 07/01/82 THROUGH 06/30/83

DOLLAR AMOUNT REQUESTED (Omit cents)

PERSONNEL (Applicant organization only) (See instructions)		TIME/EFFORT				
NAME	TITLE OF POSITION	%	Hours per Week	SALARY	FRINGE BENEFITS	TOTALS
Cabot, Myles C.	Principal Investigator	50	20	15,000	3,000	18,000
Welsh, Clement J.	Research associate	50	20	8,570	1,930	10,500
To be named	Technician	100	40	16,200	3,800	20,000
To be named	Secretarial Aide	50	20	5,200	1,300	6,500
	SUBTOTALS →			44,970	10,030	55,000

CONSULTANT COSTS (See instructions)

EQUIPMENT (Itemize)

SUPPLIES (Itemize by category)

Tissue culture ware	$4,000	Lipid enzymes, cofactors, etc.	$2,000
Culture media	3,000	Laboratory glassware	2,500
Chemicals	1,500		
Radiolabeled compounds	2,000		

15,000

TRAVEL	DOMESTIC	1,000
	FOREIGN	
PATIENT CARE COSTS	INPATIENT	
	OUTPATIENT	

ALTERATIONS AND RENOVATIONS (Itemize by category)

CONTRACTUAL OR THIRD PARTY COSTS (See instructions)

OTHER EXPENSES (Itemize by category)

TOTAL DIRECT COSTS (Also enter on page 1, item 8) → $71,000

10795-42

BUDGET E MATES FOR ALL YEARS OF SUPP T REQUESTED
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		1st BUDGET PERIOD (from page 4)	ADDITIONAL YEARS SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL (Salary and fringe benefits.) (Applicant organization only)		55,000	58,850	62,950	67,400	72,100
CONSULTANT COSTS						
EQUIPMENT						
SUPPLIES		15,000	16,200	17,500	18,900	20,400
TRAVEL	DOMESTIC	1,000	1,100	1,200	1,350	1,450
	FOREIGN					
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
CONTRACTUAL OR THIRD PARTY COSTS						
OTHER EXPENSES			500	1,000	1,000	1,000
TOTAL DIRECT COSTS		71,000	76,650	82,650	88,650	94,950

TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (Also enter on page 1, item 7) → $ 413,900

JUSTIFICATION (Use continuation pages if necessary): Briefly describe the specific functions of the personnel and consultants. For all years, justify any costs for which the need may not be obvious, such as equipment, foreign travel, alterations and renovations, and contractual or third party costs. For future years, justify any significant increases in any category. In addition, for COMPETING CONTINUATION applications, justify any significant increases over current level of support. If a recurring annual increase in personnel costs is anticipated, give percentage.

Recurring merit increases are computed at 7%. Costs under other expenses are for publication charges.

Justifications:

Technician: The person to be recruited for the position of "technician" will, initially, supplement the work load of Clement Welsh (research associate) and will later be trained to carry out the majority of work associated with cell culturing, testing morphological and biochemical markers of cellular differentiation, and analytical determinations, i.e., lipid compositional studies, thin-layer chromatography, radiolabeled tracer quantitation. This person should be self sufficient after a 6-month period. Clement Welsh has been working under my direction for 2 years; together we have coauthored papers dealing with lipid metabolism in TPA-treated HL-60 cells. His major job responsibility will be that of carrying out designed biochemical experiments and training and directing the laboratory technician. Mr. Welsh will also maintain laboratory working order and direct cell culture work.

Secretarial aide: The majority of the secretarial duties (Department of Biological Chemistry) are currently in the hands of our full-time, sole secretary. The need for an aide to help in the preparation of manuscripts, grant proposals, and other office procedures is ever apparent. We therefore request the funds to pay the salary of a secretarial aide, who would work closely with our secretary on matters regarding this proposal.

Travel: During the past few years several meetings were held with agendas closely related to my area of research (membranes, cells and phorbol esters, mechanisms of tumor promotion). Due to a lack of funds I was unable to attend these meetings, in particular the Heidelberg Conference on tumor promotion for which I was extended an invitation. Therefore, I request travel funds to attend seminars and conferences wherein I may present and discuss results with other scientists in the field of biochemistry and cell biology.

BIOGRAPHICAL SKETC

Give the following information for key professional personnel listed on page 2, beginning with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Myles C. Cabot	Biochemist	[redacted]

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
[redacted]			

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to recent representative publications, especially those most pertinent to this application. Do not exceed 2 pages.

1970-1972: Graduate Assistantship; 1969-1972, Laboratory Assistant--Western Carolina Univ., Cullowhee, NC.

1975-1976: Instructor; 1974-1976: Assistantship; 1972-1974, Fellowship--The Hebrew Univ. Jerusalem, Israel.

1976-1978: Damon Runyon-Walter Winchell Postdoc. Fellow in Cancer Research, Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee.

November 1978-Present: Scientist I, Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee (Biochemist, April 1979)

PUBLICATIONS

Cabot, M. C. and Gatt, S. *Lipase activity in rat brain.* Isr. J. Med. Sci. 11, 1194, 1975 (abstract)

Cabot, M. C. and Gatt, S. *Hydrolysis of neutral glycerides by lipases of rat brain microsomes.* Biochim. Biophys. Acta 931, 105-115, 1976.

Cabot, M. C. and Gatt, S. *Substrate specificity of the microsomal acid lipase of rat brain.* Isr. J. Med. Sci. 12, 1368, 1976 (abstract)

Cabot, M. C. and Gatt, S. *Hydrolysis of endogenous diacylglycerol and monoacylglycerol by lipases in rat brain microsomes.* Biochemistry 16, 2330-2334, 1977.

Cabot, M. C. and Gatt, S. *Rat brain microsomal lipase activity.* Adv. Exp. Med. Biol. 101, 101-111, 1978.

Cabot, M. C. and Snyder, F. *The manipulation of fatty acid composition in L-M cell monolayers supplemented with chaulmoogric acid.* Arch. Biochem. Biophys. 190, 838-846, 1978.

Cabot, M. C. and Snyder, F. *Assimilation of unnatural aliphatic moieties into complex lipids of neoplastic cells.* In: XII International Cancer Congress, Buenos Aires, Argentina, Abstracts, Workshops, Vol. 1., 1978, p. 177 (Abstract 18).

Cabot, M. C. and Gatt, S. *The hydrolysis of triacylglycerol and diacylglycerol by a rat brain microsomal lipase with an acidic pH optimum.* Biochim. Biophys. Acta 530, 508-512, 1978.

Cabot, M. C. and Snyder, F. *Manipulation of alkylglycerolipid levels in cultured cells Fatty alcohol versus alkylglycerol supplements.* Biochim. Biophys. Acta 617, 410-418, 1980.

Cabot, M. C., Welsh, C. J., Callaham, M. F., and Huberman, E. *Alterations in lipid metabolism induced by 12-O-tetradecanoyl-phorbol-13-acetate in differentiating human myeloid leukemia cells.* Cancer Res. 40, 3674-3679, 1980.
Cancer Res. 40, 3674-3679, 1980.

Cabot, M. C. and Goucher, R.R. *Chaulmoogric acid: Assimilation into the complex lipids of mycobacteria.* Lipids 16, 146-148. 1981.

Cabot, M. C. and Lumb, R. H. *The activity of a low temperature lipase in the larvae of Sarcophaga bullata (Diptera: Sarcophagidae).* Comp. Biochem. Physiol. 68B, 325-328, 1981.

Cabot, M. D. and Welsh, C. J. *Ether lipid studies in mouse C3H/10T1/2 cells and a 3-methylcholanthrene transformed clone.* Arch. Biochem. Biophys. 211, 240-244.

Cabot, M. C. and Welsh, C. J. *Fatty acid metabolism in phorbol ester-differentiating human leukemia cells.* Cancer Res. (in press)

Cabot, M. C., Welsh, C. J., and Snyder F. *Modifying the levels of ether-linked lipids in L-M cells alters growth and choline utilization.* Exptl. Cell Res. (Submitted).

OTHER SUPPORT

(USE CONTINUATION PAGES IF NECESSARY)

For each of the professionals named on page 2, list, in three separate groups: (1) active support; (2) applications pending review and/or funding; (3) applications planned or being prepared for submission. Include all Federal, non-Federal, and institutional grant and contract support. If none, state "NONE." For each item give the source of support, identifying number, project title, name of principal investigator/program director, time or percent of effort on the project by professional named, annual direct costs, and entire period of support. (If part of a larger project, provide the titles of both the parent grant and the subproject and give the annual direct costs for each.) Briefly describe the contents of each item listed. If any of these overlap, duplicate, or are being replaced or supplemented by the present application, justify and delineate the nature and extent of the scientific and budgetary overlaps or boundaries.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR:

(1) ACTIVE SUPPORT:

Investigator currently has no individual research support. He is supported under other larger projects:

A. American Cancer Society, Grant No. BC-70L; "Metabolism, Regulation and Function of Ether-Linked Glycerolipids and Their Precursors in Cancer Cells"; Dr. Fred Snyder: Senior Investigator; 20% Effort: $58,150 annual direct costs and entire period support; Purpose is to study the metabolism and regulation of ether- and ester-type glycerolipids in selected cancer cells.

B. NIH; Grant No. 5 R01 CA 11949-12; "Ether Lipids in Cancer - Enzyme Mechanisms"; Dr. Fred Snyder: Senior Investigator; 25% effort; $57,404 annual direct costs; $305,031 TDC entire period of support; Purpose is to purify key enzymes involved in the metabolism of ether linked lipids, so their kinetic properties and reaction mechanisms can be established.

C. DOE; Contract No. DE-AC05-760R00033; "Lung Surfactant, Membrane, Lipids and Energy Biohazards"; Dr. Fred Snyder: Senior Investigator; 55% effort; $223,549 annual direct costs and entire support period; Purpose of research focuses on health problems created by newer energy technologies; study the effect of chemica toxicants, carcinogens and radiation on systems being investigated.

(2) NONE.

(3) NIH; Grant Application; "Lipid Metabolism in TPA-Differentiating Leukemia"; Dr. Myles Cabot; Senior Investigator; 50% effort; $69,400 1st year annual direct costs; $220,000 TDC total support period; The purpose to establish alterations in cellular lipid composition occurring during TPA-induced differentiation of human cancer cells.

RESOURCES AND ENVIRONMENT

FACILITIES: Mark the facilities to be used and briefly indicate their capacities, pertinent capabilities, relative proximity and extent of availability to the project. Use "other" to describe facilities at other performance sites listed in Item 9, page 1, and at sites for field studies. Using continuation pages if necessary, include a description of the nature of any collaboration with other organizations and provide further information in the RESEARCH PLAN.

[x] Laboratory: My laboratory is approximately 17 x 25 ft.; it is well equipped for carrying out experiments and has two offices adjacent, one of which contains a freezer and refrigerator. The lab is located in the biochemistry department of the Medical and Health Sciences Division with analytical and preparative capacities at hand.

[ ] Clinical:

[ ] Animal:

[x] Computer: We have immediate access to computer ware.

[x] Office: My office is located adjacent to my laboratory.

[x] Other (Tissue culture): We have two culture facilities, one located directly across the hall from my laboratory. The human cell culture lab is located upstairs (approx. 45 seconds away).

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location, and pertinent capabilities of each. Liquid nitrogen refrigerator; thin-layer chromatographic equipment, including zonal scraper and spark chamber for radioassay of chromatograms; photodensitometer; preparative and analytical centrifuges; Model E ultracentrifuge, liquid scintillation spectrometer, gas-liquid chromatographs; Beckman model 324M high pressure liquid chromatography system; Beckman DU and Acta C-III spectrometer; Cahn electrobalance; protein isolation equipment. All the equipment is in excellent working order and located in the labs adjacent to my laboratory. Service contracts are maintained on all major equipment items.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultants, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

We have an on-board secretary, machine and electronics shop, glassware washing, access to electron microscopy, mass spectrometry; consultants in the areas of cell biology, cytogenetics, organic chemistry. These support services are readily available.

'USE OF DOE FACILITIES AND DOE CONTRACT REQUIREMENTS'

This research grant application includes a segment of activity that would be performed in facilities of the U. S. Department of Energy and governed by an existing contract between Oak Ridge Associated Universities (ORAU) and the DOE.

The DOE has reviewed this proposal and has concurred in ORAU conducting the described work in the DOE facilities made available for biomedical research, subject to payment to the DOE by ORAU from NIH funds of the applicable direct and indirect cost of the work (not including any charge for the use of DOE facilities) as determined by the provisions of the DOE's contract with ORAU.

It is belived that in large measure the requirements of the DOE contract parallel conditions that NIH ordinarily applies to its grants. In the event of differences between NIH grant terms and the DOE contract terms, ORAU is agreeable to meeting both to the extent that they are not in conflict, and to applying those most favorable to the United States Government where this is involved. If NIH is aware of problems that such an approach would produce or suggest, ORAU upon receipt of such advice would refer the matter to the DOE for direct resolution with NIH.

By way of general information, ORAU's contract with the DOE is a cost-type contract financed under a Government-fund account. The specific contract work is formulated in cooperation with the DOE and authorized within general guidelines in the contract. Contract terms include DOE responsibility for Government ownership and control of inventions, data, and other research products. Ownership of all equipment and facilities acquired by ORAU with DOE funds is vested in the U. S. Government at the time of acquisition. The contract also contains all the terms generally common to Government contracts of the type under which ORAU conducts research operations in Government-owned facilities.

RESEARCH PLAN

A. Specific Aims

12-O-Tetradecanoylphorbol-13-acetate (TPA) applications to mouse skin result in the production of both benign and malignant tumors if TPA treatment is preceded by application of an initiating agent (1-3). However, phorbol esters, when used in the absence of tumor initiators, induce rather than inhibit differentiation in human leukemia cells (HL-60) (4) and stimulate differentiated functions in human melanoma cells (5). A wide variety of compounds, in addition to TPA, have been shown to induce differentiation of HL-60 cells and significantly alter lipid metabolism and membrane fluidity. The results gained from this study are expected to answer the following major questions. Are similar lipid alterations common to the HL-60 cellular differentiation process without regard to the agent of induction? For example, since DMSO and retinoic acid elicit the same type of HL-60 differentiation (myeloid-granulocytic) will lipid modifications accompanying the cell transition be similar? Secondly, are the changes in lipid metabolism elicited by TPA linked to the induction of differentiation?

As phospholipids are integral components of plasma membranes and intracellular organelles, and membrane fluidity has been shown to govern membrane-bound enzyme activities, alterations in lipid metabolism occurring between control and induced cells will serve to elucidate the role of lipids in cellular differentiation and in part aid to relate functional aspects of induced differentiation to lipid compositional changes.

Specific goals of the proposed research are to: 1) establish what alterations in cellular lipid composition occur between undifferentiated cells and cells treated with the various inducers of differentiation, paying particular regard to the levels of phospholipids, sterols and triacylglycerols, and the acyl group composition of the polar lipids, and neutral lipids from whole cells or isolated plasma membranes, nuclear fractions, and mitochondrial and microsomal preparations; 2) assess uptake and utilization of labeled lipid precursors (acetate, mevalonic acid, ^{32}P, choline, ethanolamine, fatty acids, glucose) in control and induced cells as a prerequisite to guide in vitro enzymatic studies. Special emphasis will be placed on assimilation and utilization of labeled arachidonic acid, a precursor sequestered by phospholipids for the subsequent synthesis of prostaglandins. 3) Evaluate the effects of serum lipids (and their contribution to cellular lipid composition) on the induction of differentiation by utilizing cells grown in serum-free media. 4) Explore the effect of the various inducers on enzymes of lipid metabolism. Activities that will be investigated include stearol-CoA desaturase, diacylglycerol acyltransferase, enzymes of fatty acid synthesis (de novo and chain elongation), CDP-choline:1,2-diacylglycerol phosphocholinetransferase and phosphatidic acid phosphohydrolase. These enzymes play a crucial role in the maintenance of membrane fluidity and overall regulation of the levels of polar lipid membrane constituents versus neutral lipid cytosolic components. 5) Evaluate aspects of phospholipid metabolism in leukemic cell lines that are either susceptable or resistant to the action of TPA in order to explore the contributory effects of TPA as a sole factor in modulating lipid metabolism.

B. Significance

TPA, the most abundant and most active tumor-promoting agent of croton oil, has been used widely in the two-stage mouse skin carcinogenesis system (6) to study the mechanism of tumor promotion. Recent studies have shown that when tumor promoters are used singly they inhibit differentiation in murine erythroleukemia cells (7-9), mouse neuroblastoma cells (10), 3T3 fibroblasts (11), avian myoblasts (12), hamster epidermal cells (13), and avian melanocytes (14). Conversely, studies with human myeloid leukemia cells (4, 15) and human melanoma cells (5) have revealed a dramatic action of phorbol esters: one of induction rather than inhibition of cellular differentiation.

A variety of chemically unrelated compounds have been shown to induce morphological and biochemical alterations in numerous cell lines. DMSO induces erythroid differentiation in mouse erythroleukemia (Friend) cells; the morphological, biochemical, and immunological changes induced have been well characterized (16-18). The differentiation-inducing effect of DMSO has recently been demonstrated in human cells (5, 19-21) and retinoic acid has likewise been shown to induce differentiation in HL-60 cells (22). Other compounds under study that also potentiate HL-60 cell differentiation include the antibiotic, tunicamycin (23), the chemotherapeutic drug, actinomycin-D and compounds such as hypoxanthine and hexamethylene-*bis*-acetamide (21). Although these agents have not been extensively investigated, criteria for morphological and functional differentiation have been assessed in HL-60 cells following treatment. These studies suggest that HL-60 cells share common target sites for the induction of differentiation by these compounds.

Whereas a number of investigators have demonstrated that TPA addition to cell cultures causes myriad alterations in lipid metabolism, the effects of DMSO, retinoic acid, and other inducers on lipid metabolism have not been characterized. The complexity of this area can be well appreciated, for not only do tumor promoters induce differentiation in some cell lines and inhibit the differentiation process in others, preliminary studies have shown that inducer action on lipid metabolism is equally complex. Dexamethasone-induced differentiation of mouse leukemia cells produces significant changes in cellular phospholipid composition (24). Friend leukemia cells induced to differentiate with DMSO, hexamethylene-*bis*-acetamide, or sodium butyrate likewise display various lipid compositional modifications (5). Lipid metabolic studies utilizing a one-cell type -- multi-inducer system have not been carried out. A study in this vein would firstly reveal if the various inducers share common actions on lipid metabolism and secondly serve to elucidate the role of lipids in the cellular differentiation process.

Initial studies on lipid metabolism and phorbol esters revealed that TPA stimulated the early synthesis of phospholipids (26, 27). In bovine lymphocytes TPA causes an enhanced incorporation of [*Me*-^{3}H]choline into the choline-containing phospholipids (28). HeLa cells incubated with low concentrations of TPA also show a rapid increase of choline incorporation into cellular phosphatidylcholine (29). Stimulation of phospholipid metabolism, before the markers of differentiation are expressed, and enhanced triacylglycerol synthesis occur in HL-60 cells treated with TPA (30). A recent study has shown that phosphatidylcholine synthesis in HL-60 cells, via methylation of phosphatidylethanolamine, is however inhibited within 90 min of exposure to TPA (31). TPA

also evokes other lipid-associated alterations in a variety of systems: these include modifications of lipid microviscosity in lymphoblastoid cells (32), inhibition of adipose conversion in 3T3 fibroblasts (11), stimulation of surfactant secretion in alveolar type II cells (33), enhancement of phospholipase activity and prostaglandin production (34, 35), and alterations in ganglioside metabolism in human melanoma cells (5).

Does TPA directly influence lipid metabolism and do lipid modifications relate to the differentiation process? This is a question of paramount importance in the present proposal and although the query may be difficult to answer catagorically, this study will yield basic background information to clarify our understanding of the role of lipids in cell differentiation. It is likely that phospholipids play a role in the differentiation process for these membrane constituents undergo metabolic modifications before the markers for HL-60 cell differentiation are expressed. The promyelocytic lines, HL-60, KG-1, and ML-3, exhibit macrophage-like characteristics after exposure to TPA, whereas the blast lines, KG-1a and K562, are resistent to TPA-induced differentiation (36). Examining the effects of TPA on lipid metabolism in these leukemic lines will reveal whether a link exits between lipid modification and the cell differentiation process.

As can be gathered from the above examples, a considerable body of work has accrued demonstrating the action of TPA as a modulator of cell processes that effect lipid metabolism; however, only recent evidence indicates that other inducers of HL-60 cellular differentiation alter lipid metabolic processes. In work by Cooper et al. (37), inducers of myeloid differentiation were shown to inhibit sterol and phospholipid synthesis, although this information was based solely on the incorporation of radiolabeled acetate into cellular lipids. Acetate can serve as a precursor of glycerol, and be incorporated into fatty acids via de novo synthesis or by chain elongation (38). Acyl group turnover in triacylglycerols and the different classes of phospholipids should also be considered when investigating lipid metabolism via labeling techniques. There are two principal mechanisms by which fatty acids are incorporated into membrane phospholipids: de novo synthesis resulting in a net increase of phospholipid (39), and exchange reactions in which there is no increase in the amount of phospholipid (40, 41). One type of exchange reaction is catalyzed by

manipulating the fatty acid (45-48), fatty alcohol (49), or phospholipid polar head group composition (50, 51) of membrane phospholipids. Alterations accompanying the lipid modification of cellular membranes have been observed in lipid metabolism (51, 52) membrane-bound enzyme activities (53-56), cell growth (56, 57), and membrane fluidity (58, 59). These findings are supportive of the important functional role of specific lipid components in biological membranes of intact cells, and mark the significance of employing serum-free growth media and lipid supplementation to establish what lipid criteria are essential for the induction of differentiation.

The evidence that retinoids as well as tumor promoters enhance deacylation of cellular phospholipids and stimulate prostaglandin production (35) implies that essential fatty acids and prostaglandins play a role in cellular differentiation. In a recent study by Bonser et al. (60), DMSO treatment of HL-60 cells increased phospholipase and cyclooxygenase activities. Prostaglandin synthesis in a preadipocyte clonal line was shown to be maximal during cell proliferation and decrease dramatically at adipose conversion (61). Ziboh et al. (62) have shown a marked increase in the biosynthesis of $PGF_{2\alpha}$ in rapidly proliferating chloroleukemia cells, whereas no significant increase in $PGF_{2\alpha}$ occurred in quiescent hyperplastic marrow cells. Homma et al. (63) showed that differentiated mouse myeloid leukemia cells, when labeled with [^{14}C]arachidonic acid, synthesized and released prostaglandins, whereas untreated cells did not. These results strongly suggest that prostaglandins play a role in the macrophage-granulocytic differentiation process of myeloid leukemia cells. The levels of polyunsaturated fatty acids can be controlled by growing cells in serum-free media thus limiting the availability of precursors for prostaglandin production. The lipids required (i.e., essential fatty acids and prostaglandins) in the differentiation process have hitherto not been catagorized. In serum-free systems, cells that are "essential fatty acid-poor" can be treated with low levels of arachidonic acid to test the effects of membrane modification on the potentiation of cellular differentiation.

Numerous investigations have shown that chemical supplements can elicit human leukemia cell differentiation. The effect of these inducers provides morphological and biochemical markers for correlating cell differentiation with modifications of lipid metabolism. In this regard, a cell system in which markers for differentiation can be followed provides a model for studying lipid-related biochemical events during the differentiation process. Human myeloid leukemia cells are extremely useful for such studies; the cells can be induced to differentiate by a select number of compounds. Lipids are important components of cellular membranes that play a role in fluidity, regulation of enzyme activity, transport, and structure; membranes are prime targets for TPA (64-66). The fact that TPA, DMSO, and retinoic acid induce like morphological and biochemical alterations in HL-60 cells, suggests that these cells share common target sites for the induction process. Although several investigations have suggested that the cell surface plays an important role in mechanisms associated with differentiation (67, 68), there are few reports on changes of membrane components during cellular differentiation. The relationship of lipids, their compositional and metabolic aspects, to cellular differentiation has received little attention, mainly because of the lack of an appropriate system wherein modifications of lipid metabolism can be correlated and their relevancy established in tandum with the appearance of differentiation markers.

C. Preliminary Studies

Our initial results that document dramatic alterations in lipid metabolism occurring in TPA-differentiated human myeloid leukemia cells have been published (30). The data and description of these experiments can been seen in the attached reprint (Appendix I). Briefly, this work shows that TPA-induced differentiation of HL-60 cells is accompanied by a stimulation of phospholipid metabolism before the markers (attachment, morphological transformation, lysozyme levels) of differentiation are expressed, enhanced incorporation of acetate into free fatty acids and neutral lipids, an increase in the amount of cellular triacylglycerols, and a selective incorporation of [1-^{14}C]hexadecanol into triacylglycerols and their ether-containing analog, alkyldiacylglycerols. Our more recent studies (Cabot and Welsh, Cancer Res., in press, Appendix I) have revealed that TPA treatment of HL-60 cells has a profound effect on fatty acid metabolism. Most notably we found that cells treated with TPA for 48 hr show a marked decrease in stearoyl-CoA desaturase activity. Also, acyl group analyses of lipids from untreated and TPA-treated cells showed that there were no differences in the fatty acid profiles of phospholipids, although marked differences occurred in the acyl group composition of triacylglycerols between control and differentiated cells. Thus it appears that HL-60 cells, differentiated by TPA, can maintain membrane fluidity parameters that are crucial to cellular function. Our studies on fatty acid metabolism show that TPA causes an enhancement of fatty acid labeling from [^{14}C]acetate; the mechanism of acetate incorporation into HL-60 undifferentiated and TPA-differentiated cells was not ascertained. The extent of de novo synthesis vs chain-elongation of preexisting fatty acids in induced and control cells is a point of investigation in the present proposal. In previous studies we have shown that the amount of cellular triacylglycerols is higher in TPA-differentiated cells (3.2-fold increase over control with 8×10^{-10} M TPA). This modulation in neutral lipids was also verified in experiments using radiolabeled fatty acids (precursors of acyl moieties). In cells incubated with labeled stearic acid, the percent distribution of lipid radioactivity was highest in the phospholipids (controls), whereas in TPA-supplemented cells, substantially more label was associated with triacylglycerols.

We have recently initiated experiments utilizing DMSO and retinoic acid as induction agents. The cells were cultured in media containing serum and incubated in the presence of inducer for 6 days. Our preliminary data show that the acyl group composition of phospholipids is altered, compared with undifferentiated cells. Differences were also found in the acyl group composition of the triacylglycerols between control and induced cells. Although triacylglycerols are not major membrane components, they may serve as fatty acid stores. This is interesting in light of the fact that DMSO has been shown to alter membrane fluidity in HL-60 cells (69).

We have been successful in culturing HL-60 cells in serum-free media, according to the procedure described by Breitman et al. (44). The cells are currently in their 45th passage, and their fatty acid composition is relatively simple compared to cells grown in serum-rich media. The most marked change is the near absence of polyunsaturates; 16:0 + 16:1 + 18:1 account for >80% of the total acyl groups in phospholipids and triacylglycerols. Induction of differ-

entiation in these cells by TPA, DMSO, and retinoic acid is currently being assessed by morphological and biochemical criteria. Early results are encouraging with the promise that these cells, with a comparatively simple lipid composition, will complement studies on lipid requirements for cellular differentiation. The action of TPA on HL-60 cells grown in serum-free media has not yet been assessed. We have conducted morphological evaluations of these cells (1.6×10^{-8} M TPA, 48 hr) and have shown the population to consist primarily of macrophage-like cells (>70%).

I was trained in the area of lipid biochemistry, and more specifically, lipid enzymology. The pursuit of these disciplines has given me considerable experience in the fields of lipid metabolism, membrane biochemistry (membrane-enzyme-substrate interactions, membrane modification), and tissue culture. My collaborative ties with Dr. E. Huberman (Oak Ridge National Laboratory; Argonne at present) were extremely fruitful in that my knowledge in the areas of cell biology, tumor promotion, and cellular differentiation was favorably aided. As a result of my training and current laboratory environment I feel well qualified to pursue these studies. The laboratory, headed by Dr. Fred Snyder, at the ORAU Medical and Health Sciences Division, has been a forerunner in the area of lipid metabolism. The facility is well equipped for carrying out the specialized analyses that are required to explore all facets of lipid biochemistry.

D. Methods

1. Cell culture and induction of differentiation

a) Cell culture

The HL-60 cells (70), which we are using, were originally provided by Dr. R. C. Gallo, National Cancer Institute, Bethesda, Maryland, and we obtained the cells directly from Dr. E. Huberman, Biology Division, Oak Ridge National Laboratory. The KG-1 and K562 leukemia lines will be provided by Dr. B. Lozzio, University of Tennessee Memorial Research Center, Knoxville, and Dr. D. Golde, UCLA School of Medicine, Los Angeles, CA. Cells will be maintained in bacterial plastic petri dishes (No. 1007, Falcon) or cultured in 75 cm^2 Falcon flasks in an atmosphere of 5% CO_2 in air at 37°C using RPMI-1640 medium containing 20% fetal calf serum and supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml). Serum-free cells will be grown in RPMI-1640 media containing insulin and transferrin as described by Breitman et al. (44). In most experiments the cells will be seeded at $2-2.5 \times 10^6$ cells/flask and treated with an inducer the same day or 24 hr later.

b) Induction of differentiation

Culture medium will be used to make serial dilutions of a TPA-DMSO solution and added to the cells to provide the desired concentrations of TPA (10^{-10} to 10^{-8} M). By this method DMSO does not exceed 0.01% in the growth flasks, and the concentration is therefore too low to induce differentiation. This procedure has been routinely employed (4, 30). Cell incubations, in the presence of TPA, will be carried out for 48-72 hr.

Terminal differentiation induced by DMSO (19) will be carried out by incubating cells for 6 days in media containing 1.25% DMSO, and in a like manner for cells grown in serum-free media (44). Retinoic acid (all-trans-retinoic acid) will be used to induce differentiation following the procedure outlined by Breitman et al. (44). Briefly, cells will be incubated with retinoic acid, 1×10^{-6} M, for 6 days. Retinoic acid will first be dissolved in 95% ethanol and serial dilutions into growth media made so that the ethanol concentration is no higher than 0.1% in the culture flask; control flasks will receive ethanol only. HL-60 cellular differentiation induced by actinomycin-D, hexamethylene-bis-acetamide, and hypoxanthine, will be carried out according to established protocol (21). Cells will be incubated in the presence of actinomycin-D (5.0 ng/ml) for 6 days; a media change at 4 days, replaced with fresh media containing inducer, is required. Hexamethylene-bis-acetamide (2 mM) and hypoxanthine (5 mM) induction occurs likewise over a 6-day incubation period.

c) Assessment of differentiation

Given that there is some degree of variability between cells grown in various laboratories, differentiation will be assessed periodically by performing differential counts on Wright-stained preparations and by evaluation of established markers for myeloid differentiation. Morphological differentiation will be based on the percentage of myelocytes, metamyelocytes, and banded and segmented neutrophils vs. the control population that consists primarily of promyelocytes (90%). TPA-induced differentiation will be assessed by the percentage of macrophage-like cells resulting at the end of treatment. Other criteria will include cell attachment to the substratum, and cessation of growth. Biochemical markers for differentiation include NBT dye reduction (21, 71), phagocytosis of Candida albicans (21), lysozyme release (72), and specifically in the case of TPA, acid phosphatase activity for the macrophage differentiation produced (73).

d) Isolation of subcellular fractions

Plasma membranes will be isolated following a published protocol specific for HL-60 cells (69). Nuclei will be isolated according to a procedure described for guinea pig polymorphonuclear leukocytes (74). This procedure employs the homogenization of cells in a medium that is slightly hypotonic followed by immediate restoration of isotonicity. By this method the integrity of other subcellular organelles is maintained. The purity of the subcellular fractions will be established by assay of classical marker enzymes: NADPH cytochrome c reductase for microsomes (75); monoamine oxidase for mitochondria (76); β-N-acetylglycosaminidase for lysosomes (77) 5′-nucleotidase (78) and (Na^+ plus K^+)-ATPase (79) for plasma membranes; and lactate dehydrogenase for the soluble fraction (80).

2. Lipid analyses

Cellular lipid composition will be compared in uninduced and induced leukemia cells with special regard to amount and types of phospholipids, (choline-,

ethanolamine-, serine-, and inositol-containing), neutral lipids (sterols, triacylglycerols, alkyldiacylglycerols, cholesterol esters), and acyl group compositions of the glycerolipids. Lipids will be extracted from control and differentiated cells by a modified method of Bligh and Dyer (81) in which the methanol contains 2% glacial acetic acid. Both neutral and polar lipids can be resolved by thin-layer chromatography in a variety of solvent systems (82). Neutral lipids will be separated on layers of Silica Gel G and phospholipids resolved on Silica Gel HR. A comparison of R_f values with commercial standards will be used for identification of lipid classes. Quantitative analysis of thin-layer-resolved lipids is accomplished by H_2SO_4 charring of the chromatoplates (pre-run in diethylether, 100%) at 200°C followed by photodensitometry according to Privett et al. (83). This method will be used to determine the contributing amounts of sterols, triacylglycerols, alkyldiacylglycerols, and cholesterol esters to the total lipid fraction. Total phospholipids are quantitated by measuring phospholipid phosphorus (84), and in a like manner, phosphorus of the various phospholipid classes will be determined directly by analysis of H_2SO_4-charred lipids. Acyl group composition will be analyzed in phospholipids (total and individual classes) and triacylglycerols by first isolating lipids on preparative thin-layer chromatographic plates. Phospholipids, triacylglycerols, alkyldiacylglycerols, and cholesterol esters are separated on chromatoplates developed in hexane/diethylether/acetic acid (80:20:1). Methyl esters will be prepared by refluxing the lipids in methanol containing 2% H_2SO_4 at 100°C (85) and analyzed by gas-liquid chromatography as described by Blank and Snyder (86) and Blank et al. (53). Quantitation will be based on weight•percent using a Hewlett-Packard recording integrator. Additionally, the positional specificity of the fatty acids in the major phospholipid classes will be determined by gas-liquid chromatography after treatment of the isolated phospholipids with phospholipase A_2 (57).

3. Lipid metabolic studies

a) Fatty acid metabolism

Fatty acid desaturase activity in undifferentiated and differentiated cells will be assayed in whole cells (53) or cell-free homogenates (87). Briefly, control cells and induced cultures will be incubated with [1-^{14}C]stearic acid for 1-2 hr. Methyl esters are then prepared from the lipid extracts of harvested, washed cells, and separated according to the degree of unsaturation on $AgNO_3$-impregnated thin-layer plates (88). Bands corresponding to monoenes will be visualized with 2′,7′-dichlorofluorescein and scraped for radioactive analysis by liquid scintillation spectrometry. The cell free system will consist of microsomes (0.2-1.0 mg protein) from control or induced cells, 0.1 M Tris-HCl buffer, pH 7.2, 30-70 µM [1-^{14}C]steaoyl-CoA, and 0.4 mM NADPH. After a 5-min incubation at 30°C, the reaction is saponified, acidified, and methylated for thin-layer analysis. An alternate method of assay employs replacement of NADPH with an NADPH-generating system (1 mM $NADP^+$, 3 mM $MgSO_4$, 10 mM glucose-6-P, excess glucose-6-P dehydrogenase). To evaluate the effects of differentiation on both fatty acid elongation and desaturation, control and

induced cells will be incubated with [1-^{14}C]palmitic acid for 1 hr and the metabolic products in the lipid extracts from cells analyzed by combined gas-liquid chromatography and collection of $^{14}CO_2$ as described by Blank et al. (53).

Immature leukemic blast cells contain acetyl-CoA carboxylase and are capable of fatty acid synthesis de novo; however, human leukocytes lack acetyl-CoA carboxylase and incorporate acetate into fatty acids via chain elongation (38, 89). This interesting contrast between immature and differentiated cells can be employed as a marker for differentiation. For this reason, fatty acid synthesis, de novo, and by chain elongation will be assayed in control and differentiated prototypes. Intact cells will be incubated in regular media containing serum, or for short labeling periods, washed and resuspended in serum-free media. [1-^{14}C]Acetate (10 μCi/flask) will be introduced and incubated for periods up to 24 hr. Aliquots of cellular total lipids are then hydrolyzed in 2 N methanolic KOH for 2 hr at 70°C and subjected to Schmidt degradation by the method of Brady et al. (90). This technique, which removes the cabonyl carbon of fatty acids (C-1) is used to distinguish between de novo fatty acid synthesis and chain elongation. Radioactivity in the total fatty acid fraction will then be compared with the percentage evolved as $^{14}CO_2$ from the C-1 position. The overall capacity of undifferentiated and induced cells to synthesize fatty acids will be tested in cell-free extracts using the radioisotopic method described for determination of fatty acid synthase activity (91).

b) Acyl group modification of cellular lipids

The effects of altering membrane fluidity on the potentiation of induction will be tested on cells grown in media containing supplemented fatty acids. Cells grown in serum-containing or serum-free media can be manipulated to favor acyl group enrichment with a particular fatty acid (48, 56, 57, 92-96). Culturing cells in serum-free media drastically alters the acyl group composition of the glycerolipids (see Preliminary Studies). Because the effects of serum removal on cell differentiation are not well understood, we will carry out our initial supplementation studies on cells grown in serum-containing media. Thus, any differences noted in the course of induction can be ascribed to the enrichment rather than a combination of acyl group enrichment and serum deprivation. We have considerable experience in the area of membrane modification; therefore, we anticipate that a 24-hr exposure to fatty acids (3-10 μg/ml growth media) will be sufficient to accomplish enrichment. Fatty acids (stearic and arachidonic) will be introduced as the Na^+ soaps and phospholipid acyl groups will be analyzed to determine the modifications achieved (57). Once a dose-response relationship and toxicity levels have been established, the enriched cells will be exposed to inducers, and the time course for differentiation, based on morphological and biochemical criteria, will be determined. Breitman et al. (44) have shown that HL-60 cells grown in serum-free media can be induced to differentiate in the presence of 1.2% DMSO; we have preliminary data that shows the same occurs with TPA (1.6×10^{-8} M). Therefore, we will employ cells grown in serum-free media as controls to correlate the inductive effects of DMSO and TPA on cells cultured in serum-free media containing either stearic or arachidonic acids. Radiolabeled

stearic and arachidonic acids, incubated with cells for 24 hr, will be used as tracers to determine the distribution of fatty acid supplements in plasma membrane and nuclear fractions.

c) Phospholipid metabolism in TPA-susceptable and TPA-resistant lines

Because modifications of phospholipid metabolism are the earliest lipid changes occurring after TPA supplementation of HL-60 cells, we will evaluate the utilization of ^{32}P,[^{14}C]choline, and [^{3}H]methionine for the metabolism of cellular phospholipids. For these experiments we will utilize HL-60 and KG-1 cell lines that undergo macrophage differentiation after TPA exposure, and TPA resistant cells (K562 and KG-1a) that do not express macrophage characteristics in the presence of TPA (36). Information from these experiments will be useful in determining whether TPA exerts direct action on phospholipid metabolism or if lipid modifications are linked to the differentiation process.

d) Metabolism of prostaglandin precursors

Our studies on prostaglandin metabolism in undifferentiated and differentiated cells will center on the assimilation of labeled prostaglandin precursors (linoleic and arachidonic acids), and the levels of phospholipase A_2 activity in cells. [1-^{14}C]Linoleic acid or [1-^{14}C]arachidonic acid will be incubated with control and induced cells to establish the time course of uptake and the distribution of radioactivity in cellular phospholipids (choline-, ethanolamine-, inositol-, and serine-containing glycerophospholipids) and triacylglycerols. The level of phospholipase A_2 activity will be assayed in control and differentiated cells prelabeled with arachidonic acid according to the procedure described by Bonser et al. (60). Briefly, cell cultures will be incubated with [1-^{14}C]arachidonic acid (0.1 µCi/ml culture media). Washed labeled cells are then resuspended in assay buffer (50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1.4 mM $CaCl_2$, and 0.7 mM $MgCl_2$). The 1-ml cell suspensions (5 x 10^7 cells/ml) are then incubated with or without 10 µM calcium ionophore A_{23187} for 5 min at 37°C. Solvent extractions of the reaction mixture will then be assayed for free arachidonate by thin-layer chromatography. Prelabeled cells will first be analyzed to determine the distribution of labeled arachidonate in each of the phospholipid classes. By this means we can correlate the appearance of free arachidonic acid with depletion of radioactivity in the particular phospholipid classes. The contribution of phospholipase C and neutral glyceride lipase to arachidonic acid release (97, 98) and prostaglandin production will be assessed by measuring radioactivity in phosphatidylinositol and diacylglycerol fractions from undifferentiated and differentiated cells. Another factor that may influence the metabolism of prostaglandin precursors in control and inducer-treated cells is the activity of the Δ6 desaturase, an enzyme that is lost in transformed cells (99). The extent to which this desaturase influences prostaglandin production will be evaluated in treated (TPA, DMSO, retinoic acid) and control cells by determining the distribution of radioactivity in fatty acids from cells incubated with labeled linoleate 18:2 (n-6).

e) In vitro enzymic assays

Diacylglycerol acyltransferase will be assayed using microsomes as the enzyme source isolated from control and differentiated cells according to established procedures (100, 101). Measurement of the enzyme activity will also be carried out utilizing endogenous diacylglycerols. Briefly, microsomal fractions will be incubated with phospholipase C (102) to generate endogenous, membrane-bound diacylglycerols. These microsomes (0.2-0.4 mg protein) will then be incubated at 37°C in medium containing 50 mM NaF, 2.0 mM dithiothreitol, bovine serum albumin (5 mg/ml) and 0.1 mM [1-^{14}C]palmitoyl-CoA. Upon termination the lipids will be extracted from the reaction mixture and radiolabeled triacylglycerols separated by thin-layer chromatography. Phosphatidic acid phosphohydrolase activity will be measured by adding an exogenous dispersion of phosphatidic acid to the microsomal or soluble fraction from control or differentiated cell preparations (103, 104). An alternate assay method will employ microsomal-bound [^{14}C]phosphatidate as substrate (103). Cholinephosphotransferase (CDP-choline:1,2-diacylglycerol phosphocholinetransferase) will be assayed by incorporation of phospho-[Me-^{14}C]choline into phosphatidylcholine from CDP-[Me-^{14}C]choline. The procedure described by Weiss et al. (105) and modified by Vance and Burke (106) will be used in the assay of this enzyme.

4. Priorities

The priorities of our proposed work will be essentially according to the order outlined (pp.18-22). However, preliminary data will be obtained through lipid compositional studies of cells treated with the various induction agents, and these results will direct future project decisions so that emphasis can be placed on mechanistic studies such as subcellular site of action, lipid enzymology, and the role of serum and essential fatty acids.

Timetable

First year: We will concentrate our efforts on determining the lipid compositional changes associated with the induction of differentiation utilizing the various agents. Close scrutiny will be given to assessment of differentiation (morphological and biochemical criteria) so that the dynamics of lipid alterations can be correlated with the inducer and degree of differentiation. Recruitment and training of a technician will be undertaken during the first 6 months.

Second year: Studies will be initiated using cells grown in serum-free media and results compared with data derived from serum-grown cells in order to assess the effect of serum deprivation on the induction of differentiation. All lipid compositional studies will be completed by the end of year 2. We will start, and continue throughout the remainder of entire grant period, evaluation of lipid metabolism in human leukemia cell lines that are either resistant or susceptible to TPA action.

Third year: We will initiate and complete studies utilizing labeled lipid precursors to determine turnover rates of the various components of cellular lipids (acyl groups, polar head groups, glycerol backbone). These data, together with the compositional studies will set the groundwork for mechanistic studies to pinpoint enzymatic modifications. Uptake and utilization of labeled lipid precursors will be closely assessed for all the experimental induction agents.

Fourth year: We will initiate aspects of fatty acid metabolism: desaturation, elongation, de novo synthesis. Enzymatic studies will be continued to include examination of those activities outlined in Aims. We will initiate and complete a specific study of linoleic and arachidonic acid metabolism inclusive of phospholipase A_2 activity and prostaglandin production.

Fifth year: The effects of membrane modification on cellular differentiation will be studied by utilizing cells grown in serum-free media with or without supplemented fatty acids. As the specific format for experiments outlined may lend itself to studies aimed at the involvement of plasma and nuclear membrane lipids and differentiation, we will, throughout the entire funding period, explore the effects of induction on lipid composition of subcellular fractions to determine which subcellular fractions are targets for change.

E. Human subjects, derived materials or data

The biological materials used in this study consist of cultured cell lines of human origin that were established in 1977. Our cell culture facility is satisfactorily equipped to handle and dispose of this material in a safe fashion.

F. Laboratory animals

None.

G. Consultant

We have working arrangements with a number of scientists outside our group: cell biologists and toxicologists (Dr. E. Huberman, Argonne, Chicago), organic chemists (Dr. C. Piantadosi, University of North Carolina, Chapel Hill), and mass spectroscopy (Dr. W. Rainey, Oak Ridge National Laboratory). Dr. B. Lozzio, University of Tennessee Memorial Reseach Center, has graciously agreed to provide leukemia cell lines that are either resistant or susceptible to TPA action. These scientists will be helpful in solving some of the specialized problems that may arise.

Dr. Gayle Littlefield, head of the Cytogenetics Department of our Division, has agreed to aid in the morphological assessment of inducer-treated cells. A curriculum vitae for Dr. Littlefield is Appendix II.

H. Consortium arrangements or formalized collaborative agreements

None.

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CHECKLIST

This is the required last page of the application.

Check the appropriate boxes and provide the information requested.

TYPE OF APPLICATION:

[X] NEW application *(This application is being submitted to the PHS for the first time.)*

[ ] COMPETING CONTINUATION of grant number: _____.
(This application is to extend a grant beyond its original project period.)

[ ] SUPPLEMENT to grant number: _____.
(This application is for additional funds during a funded project period.)

[ ] REVISION of application number: _____.
(This application replaces a prior version of a new, competing continuation or supplemental application.)

[ ] Change of Principal Investigator/Program Director.
Name of former Principal Investigator/Program Director: _____.

ASSURANCES IN CONNECTION WITH:

Civil Rights	Handicapped Individuals	Sex Discrimination	Human Subjects General Assurance *(If applicable)*	Laboratory Animals *(If applicable)*
[X] Filed	[X] Filed	[X] Filed	[ ] Filed	[X] Filed
[ ] Not filed	[ ] Not filed	[ ] Not filed	[ ] Not filed	[ ] Not filed

INDIRECT COSTS:

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHEW Regional Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHEW Guide for Establishing Indirect Cost Rates*, and submit it to the appropriate DHEW Regional Office. Indirect costs will not be paid on foreign grants, construction grants, and grants to individuals, and usually not on grants in support of conferences.

[X] DHEW Agreement Dated: December 9, 1980.

--- % Salary and Wages or 55.3 % Total Direct Costs.

Is this an off-site or other special rate, or is more than one rate involved? [ ] YES [X] NO
Explanation: _____

[ ] DHEW Agreement being negotiated with _____ Regional Office.

[ ] No DHEW Agreement, but rate established with _____, Date _____.

[ ] No Indirect Costs Requested.

1079566

Oak Ridge
Associated
Universities

September 18, 1981

Dr. William R. Bibb, Director
Research Division
Department of Energy
Oak Ridge, TN 37830

Subject: DRAFT APPLICATION TO NIH FOR A PROJECT ENTITLED "LIPID METABOLISM AND DIFFERENTIATORS OF LEUKEMIA"

Dear Dr. Bibb:

Enclosed are three copies of a draft application to NIH for a project entitled "Lipid Metabolism and Differentiators of Leukemia" to be carried out under the direction of Dr. Myles C. Cabot. If approved, this work will be carried out under procedures and policies already established between ORAU and the DOE for work in facilities owned by DOE. Should any questions arise during your review of this proposal please do not hesitate to call Dr. Cabot at 6-3122.

Formal copies of this grant application should be forwarded to NIH no later than October 26, 1981.

Sincerely,

Philip L. Johnson
Executive Director

RYAN:br

Enclosures

20.88.81

1079567

FORM APPROVED
O.M.B. NO. 68-R0249

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

GRANT APPLICATION

FOLLOW INSTRUCTIONS CAREFULLY

LEAVE BLANK		
TYPE	ACTIVITY	NUMBER
REVIEW GROUP		FORMERLY
COUNCIL/BOARD (Month, year)		DATE RECEIVED

1. TITLE OF APPLICATION (Do not exceed 56 typewriter spaces)
LIPID METABOLISM AND DIFFERENTIATORS OF LEUKEMIA

2. RESPONSE TO SPECIFIC PROGRAM ANNOUNCEMENT [x] NO [ ] YES (If "YES," state RFA number and/or announcement title)

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (Last, first, middle)
CABOT, MYLES C.

3b. SOCIAL SECURITY NUMBER
[redacted]

3c. MAILING ADDRESS (Street, city, state, zip code)
Medical and Health Sciences Division
Oak Ridge associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

3d. POSITION TITLE
Biochemist

3e. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
Biological Chemistry

3f. TELEPHONE (Area code, number and extension)
615 576-3-22

3g. MAJOR SUBDIVISION
Medical and Health Sciences Division

4. HUMAN SUBJECTS, DERIVED MATERIALS OR DATA INVOLVED
[xx] NO [ ] YES (If "YES," form HEW 596 required)

5. RECOMBINANT DNA RESEARCH SUBJECT TO NIH GUIDELINES
[xx] NO [ ] YES

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)
From: July 1, 1982 Through: June 30, 1987

7. TOTAL DIRECT COSTS REQUESTED FOR PROJECT PERIOD (from page 5)
$376,500

8. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD (from page 4)
$64,500

9. PERFORMANCE SITES (Organizations and addresses)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

10. INVENTIONS (Competing continuation application only)
Were any inventions conceived or reduced to practice during the course of the project?
[x] NO [ ] YES - Previously reported
[ ] YES - Not previously reported

11. APPLICANT ORGANIZATION (Name, address, and congressional district)
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37839

Third Congressional District

12. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT (See instructions)

Code [2 0] Description:
(Medical and Health Sciences Division)

13. ENTITY IDENTIFICATION NUMBER
162047681A1

14. TYPE OF ORGANIZATION (See instructions)
[xx] Private Nonprofit
[ ] Public (Specify Federal, State, Local):

15. OFFICIAL IN BUSINESS OFFICE TO BE NOTIFIED IF AN AWARD IS MADE (Name, title, address and telephone number.)
William F. Countiss
Head, Office of Fiscal Services
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830 (515 576-3056)

16. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Name, title, address and telephone number)
Philip L. Johnson
Executive Director
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830 (615, 576-3300)

17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a (In ink. "Per" signature not acceptable)

DATE

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense. (U.S. Code, Title 18, Section 1001.)

SIGNATURE OF PERSON NAMED IN 16 (In ink. "Per" signature not acceptable)

DATE

PHS-398
Rev. 10.79

1079568

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

LEAVE BLANK

PROJECT NUMBER

ABSTRACT OF RESEARCH PLAN

NAME AND ADDRESS OF APPLICANT ORGANIZATION (Same as Item 11, page 1)
Oak Ridge Associated Universities, P. O. Box 117
Oak Ridge, Tennessee 37830

TITLE OF APPLICATION (Same as Item 1, page 1)
LIPID METABOLISM AND DIFFERENTIATORS OF LEUKEMIA

Name, Title and Department of all professional personnel engaged on project, beginning with Principal Investigator/Program Director

Mylec C. Cabot, Ph.D., Biochemist, Biological Chemistry, Medical and Health Sciences Division

ABSTRACT OF RESEARCH PLAN: Concisely describe the application's specific aims, methodology and long-term objectives, making reference to the scientific disciplines involved and the health-relatedness of the project. The abstract should be self-contained so that it can serve as a succinct and accurate description of the application when separated from it. DO NOT EXCEED THE SPACE PROVIDED.

The potent tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), and the compounds dimethyl sulfoxide (DMSO) and retinoic acid have been shown to induce morphological and functional differentiation of human leukemia cells (HL-60). Significant modifications of lipid metabolism accompany the induction of differentiation, although it is not known whether the lipid changes arrising during this process are common to differentiation or varied and dependent upon the type of inducer employed. We have shown that treatment of HL-60 cells with TPA elicits stimulation of phosphatidylcholine metabolism, increases triacylglycerol synthesis, decreases stearoyl-CoA desaturase activity and modifies the fatty acid composition of triacylglycerols. By using the tumor promoter, TPA, polar planar compounds (DMSO, hexamethylene bisacetamide), retinoic acid, and certain purines, particularly hypoxanthine and the chemotherapeutic agent, actinomycin-D, we will be able to correlate the dynamics of lipid metabolism with cellular differentiation. HL-60 cells will be cultured either in the presence or absence of serum, exposed to inducers of differentiation and rigorously characterized using morphological and biochemical criteria. Total lipids isolated from control and differentiated cells will be extensively analyzed. Alterations noted in lipid composition of cells will guide the format of experiments designed to determine the mechanisms responsible for the changes, i.e., uptake rates and assimilation of labeled precursors, activities of the synthetic and degradative enzymes. A major thrust of this study will be to compare inducer effect on cells grown in the presence and absence of serum. The latter method of cell culture greatly modifies the acyl group composition cellular lipids and will potentiate studies correlating inducer capabilities with lipid composition; namely, what role do essential fatty acids and membrane composition play in the process of induction. The ultimate objective is to more fully explore and gain insight into the relationship of lipids to cellular diffentiation.

LABORATORY ANIMALS INVOLVED. Identify by common names. If none, state "none"

TABLE OF CONTENTS

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 5a, 5b. Type the name of the Principal Investigator/Program Director at the top of each printed page and each continuation page.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECT

DETAILED BUDGET FOR FIRST 12 MONTH BUDGET PERIOD
DIRECT COSTS ONLY

FROM 07/01/82 THROUGH 06/30/83

DOLLAR AMOUNT REQUESTED (Omit cents)

PERSONNEL (Applicant organization only) (See instructions)		TIME/EFFORT		SALARY	FRINGE BENEFITS	TOTALS
NAME	TITLE OF POSITION	%	Hours per Week			
Cabot, Myles C.	Principal Investigator	50	20	15,000	3,000	18,000
Welsh, Clement J.	Research associate	50	20	8,570	1,930	10,500
To be named	Technician	100	40	16,200	3,800	20,000
	SUBTOTALS →			39,770	8,730	48,500

CONSULTANT COSTS (See instructions)

EQUIPMENT (Itemize)

SUPPLIES (Itemize by category)

Tissue culture ware	$4,000	Lipid enzymes, cofactors, etc.	$2,000
Culture media	3,000	Laboratory glassware	2,500
Chemicals	1,500		
Radiolabeled compounds	2,000		

15,000

TRAVEL	DOMESTIC	1,000
	FOREIGN	
PATIENT CARE COSTS	INPATIENT	
	OUTPATIENT	

ALTERATIONS AND RENOVATIONS (Itemize by category)

CONTRACTUAL OR THIRD PARTY COSTS (See instructions)

OTHER EXPENSES (Itemize by category)

TOTAL DIRECT COSTS (Also enter on page 1, Item 8) → $64,500

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BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		1st BUDGET PERIOD (from page 4)	ADDITIONAL YEARS SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL (Salary and fringe benefits.) (Applicant organization only)		48,500	51,900	55,500	59,400	63,600
CONSULTANT COSTS						
EQUIPMENT						
SUPPLIES		15,000	16,200	17,500	18,900	20,400
TRAVEL	DOMESTIC	1,000	1,110	1,200	1,350	1,450
	FOREIGN					
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
CONTRACTUAL OR THIRD PARTY COSTS						
OTHER EXPENSES			500	1,000	1,000	1,000
TOTAL DIRECT COSTS		64,500	69,700	75,200	80,650	86,450

TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (*Also enter on page 1, item 7*) → $376,500

JUSTIFICATION (*Use continuation pages if necessary*): Briefly describe the specific functions of the personnel and consultants. For all years, justify any costs for which the need may not be obvious, such as equipment, foreign travel, alterations and renovations, and contractual or third party costs. For future years, justify any significant increases in any category. In addition, for COMPETING CONTINUATION applications, justify any significant increases over current level of support. If a recurring annual increase in personnel costs is anticipated, give percentage.

Recurring salary increases computed at 7%. Costs under other expenses are for publication charges.

Technician justification: The person to be recruited for the position of "technician" will, initially, supplement the work load of Clement Welsh (research associate) and will later be trained to carry out the majority of work associated with cell culturing, testing morphological and biochemical markers for cellular differentiation and analytical determinations, i.e., lipid compositional studies, thin-layer chromatography, radiolabeled tracer quanitation. This person should be self-sufficient after a 6-month period.

BIOGRAPHICAL SKETCH

Give the following information for key professional personnel listed on page 2, beginning with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Myles C. Cabot	Biochemist	[redacted]

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
[redacted]			

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to recent representative publications, especially those most pertinent to this application. Do not exceed 2 pages.

1970-1972: Graduate Assistantship; 1969-1972, Laboratory Assistant--Western Carolina Univ., Cullowhee, NC.

1975-1976: Instructor; 1974-1976: Assistantship; 1972-1974, Fellowship--The Hebrew Univ. Jerusalem, Israel.

1976-1978: Damon Runyon-Walter Winchell Postdoc. Fellow in Cancer Research, Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee.

November 1978-Present: Scientist I, Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee (Biochemist, April 1979)

PUBLICATIONS

Cabot, M. C. and Gatt, S. *Lipase activity in rat brain.* Isr. J. Med. Sci. 11, 1194, 1975 (abstract)

Cabot, M. C. and Gatt, S. *Hydrolysis of neutral glycerides by lipases of rat brain microsomes.* Biochim. Biophys. Acta 931, 105-115, 1976.

Cabot, M. C. and Gatt, S. *Substrate specificity of the microsomal acid lipase of rat brain.* Isr. J. Med. Sci. 12, 1368, 1976 (abstract)

Cabot, M. C. and Gatt, S. *Hydrolysis of endogenous diacylglycerol and monoacylglycerol by lipases in rat brain microsomes.* Biochemistry 16, 2330-2334, 1977.

Cabot, M. C. and Gatt, S. *Rat brain microsomal lipase activity.* Adv. Exp. Med. Biol. 101, 101-111, 1978.

Cabot, M. C. and Snyder, F. *The manipulation of fatty acid composition in L-M cell monolayers supplemented with chaulmoogric acid.* Arch. Biochem. Biophys. 190, 838-846, 1978.

Cabot, M. C. and Snyder, F. *Assimilation of unnatural aliphatic moieties into complex lipids of neoplastic cells.* In: XII International Cancer Congress, Buenos Aires, Argentina, Abstracts, Workshops, Vol. 1., 1978, p. 177 (Abstract 18).

Cabot, M. C. and Gatt, S. *The hydrolysis of triacylglycerol and diacylglycerol by a rat brain microsomal lipase with an acidic pH optimum.* Biochim. Biophys. Acta 530, 508-512, 1978.

Cabot, M. C. and Snyder, F. *Manipulation of alkylglycerolipid levels in cultured cells Fatty alcohol versus alkylglycerol supplements.* Biochim. Biophys. Acta 617, 410-418, 1980.

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Cabot, M. C., Welsh, C. J., Callaham, M. F., and Huberman, E. *Alterations in lipid metabolism induced by 12-O-tetradecanoyl-phorbol-13-acetate in differentiating human myeloid leukemia cells.* Cancer Res. 40, 3674-3679, 1980.
Cancer Res. 40, 3674-3679, 1980.

Cabot, M. C. and Goucher, R.R. *Chaulmoogric acid: Assimilation into the complex lipids of mycobacteria.* Lipids 16, 146-148. 1981.

Cabot, M. C. and Lumb, R. H. *The activity of a low temperature lipase in the larvae of Sarcophaga bullata (Diptera: Sarcophagidae).* Comp. Biochem. Physiol. 68B, 325-328, 1981.

Cabot, M. D. and Welsh, C. J. *Ether lipid studies in mouse C3H/10T1/2 cells and a 3-methylcholanthrene transformed clone.* Arch. Biochem. Biophys. (in press).

Cabot, M. C. and Welsh, C. J. *Fatty acid metabolism in phorbol ester-differentiating human leukemia cells.* Cancer Res. (in press)

Cabot, M. C., Welsh, C. J., and Snyder F. *Modifying the levels of ether-linked lipids in L-M cells alters growth and choline utilization.* Exptl. Cell Res. (Submitted).

OTHER SUPPORT

(USE CONTINUATION PAGES IF NECESSARY)

For each of the professionals named on page 2, list, in three separate groups: (1) active support; (2) applications pending review and/or funding; (3) applications planned or being prepared for submission. Include all Federal, non-Federal, and institutional grant and contract support. If none, state "NONE." For each item give the source of support, identifying number, project title, name of principal investigator/program director, time or percent of effort on the project by professional named, annual direct costs, and entire period of support. (If part of a larger project, provide the titles of both the parent grant and the subproject and give the annual direct costs for each.) Briefly describe the contents of each item listed. If any of these overlap, duplicate, or are being replaced or supplemented by the present application, justify and delineate the nature and extent of the scientific and budgetary overlaps or boundaries.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR:

(1) ACTIVE SUPPORT:

Investigator currently has no individual research support. He is supported under other larger projects:

A. American Cancer Society, Grant No. BC-70L; "Metabolism, Regulation and Function of Ether-Linked Glycerolipids and Their Precursors in Cancer Cells"; Dr. Fred Snyder: Senior Investigator; 20% Effort: $58,150 annual direct costs and entire period support; Purpose is to study the metabolism and regulation of ether- and ester-type glycerolipids in selected cancer cells.

B. NIH; Grant No. 5 R01 CA 11949-12; "Ether Lipids in Cancer - Enzyme Mechanisms"; Dr. Fred Snyder: Senior Investigator; 25% effort; $57,404 annual direct costs; $305,031 TDC entire period of support; Purpose is to purify key enzymes involved in the metabolism of ether linked lipids, so their kinetic properties and reaction mechanisms can be established.

C. DOE; Contract No. DE-AC05-760R00033; "Lung Surfactant, Membrane, Lipids and Energy Biohazards"; Dr. Fred Snyder: Senior Investigator; 55% effort; $223,549 annual direct costs and entire support period; Purpose of research focuses on health problems created by newer energy technologies; study the effect of chemical toxicants, carcinogens and radiation on systems being investigated.

(2) NONE.

(3) NIH; Grant Application; "Lipid Metabolism in TPA-Differentiating Leukemia"; Dr. Myles Cabot; Senior Investigator; 50% effort; $69,400 1st year annual direct costs; $220,000 TDC total support period; The purpose to establish alterations in cellular lipid composition occurring during TPA-induced differentiation of human cancer cells.

RESOURCES AND ENVIRONMENT

FACILITIES: Mark the facilities to be used and briefly indicate their capacities, pertinent capabilities, relative proximity and extent of availability to the project. Use "other" to describe facilities at other performance sites listed in Item 9, page 1, and at sites for field studies. Using continuation pages if necessary, include a description of the nature of any collaboration with other organizations and provide further information in the RESEARCH PLAN.

[X] Laboratory: My laboratory is approximately 17 x 25 ft.; it is qell equipped for carring out experiments and has two offices adjacent, one of which contains a freezer and refrigerator. The lab is located in the biochemistry department of the Medical and Health Sciences Division with analytical and preparative capacities at hand.

[ ] Clinical:

[ ] Animal:

[X] Computer: We have immediate access to computer ware.

[X] Office: My office is located adjacent to my laboratory.

[X] Other (Tissue culture): We have two culture facilities, on e located directly across the hall from my laboratory. The human cell culture lab is located upstains (approx. 45 seconds away).

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location, and pertinent capabilities of each. Lipid nitrogen refrigerator; thin-layer chromatographic equipment, including zonal scraper and spark chamber; photodensitometer; preparative and analytical centrifuges; Model E ultracentrifuge, liquid scintillation spectrometer, gas-liquid chromatographs; HPLC; Beckman DU and Acta C-III spectrometer; Cahn electrobalance; protein isolation equipment. All the equipment is in excellent working order and located in the labs adjacent to my laboratory. Service contracts are active.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultants, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

We have on board secretarial staff, machine and electronics shop, access to electron microscopy, mass spectrometry; consultants in the areas of cell biology, cytogenetics, organic chemistry. The support services are readily available.

PRINCIPAL INVESTIGATOR/PROGRAM DIREC :

DETACH AND CLIP TO THE SIGNED FACE PAGE OF THE APPLICATION

PERSONAL DATA ON

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RESEARCH PLAN

A. Specific Aims

12-O-Tetradecanoylphorbol-13-acetate (TPA) applications to mouse skin result in the production of both benign and malignant tumors if TPA treatment is preceded by application of an initiating agent (Baird and Boutwell, 1971; Hecker, 1971; Van Duuren, 1969). However, phorbol esters, when used in the absence of tumor initiators, induce rather than inhibit differentiation in human leukemia cells (HL-60) (Huberman and Callaham, 1979) and stimulate differentiated functions in human melanoma cells (Huberman et al., 1979). A wide variety of compounds, in addition to the phorbol ester tumor promoting agents, have been shown to induce cellular differentiation of HL-60 cells and signifiantly alter lipid metabolism and membrane fluidity. The results gained from this study are expected to answer the following major question. Are like lipid alterations common to the HL-60 cellular differentiation process without regard to the agent of induction? For example, since DMSO and retinoic acid elicit the same type of HL-60 differentiation (myeloid-granulocytic) will lipid modifications accompanying the cell transition be similar?

As phospholipids are integral components of plasma membranes and intracellular organelles, and membrane fluidity has been shown to govern membrane-bound enzyme activities, alterations in lipid metabolism occurring between control and induced cells will serve to elucidate the role of lipids in cellular differentiation and in part aid to relate functional aspects of induced differentiation to lipid compositional changes.

Specific goals are to establish what alterations in cellular lipid composition occur between undifferentiated cells and cells treated with the

various inducers of differentiation, paying particular regard to the levels of phospholipids, sterols and triacylglycerols, and the acyl group composition of the polar lipids, and neutral lipids from whole cells or isolated plasma membrane and nuclea fractions. The uptake and utilization of labeled lipid precursors (acetate, mevalonic acid, ^{32}P, choline, ethanolamine, fatty acids, glucose) will be assessed in control and induced cells as a prerequisite to guide in vitro enzymatic studies. Special emphasis will be placed on assimilation and utilization of labeled arachidonic acid, a precursor sequestered by phospholipids for the subsequent synthesis of prostaglandins. In turn we will utilize cells grown in serum-free media to assess the effects of serum lipids (and their contribution to cellular lipid composition) on the induction of differentiation. Studies at the enzymatic level will be carried out to explore the effect of various inducers on the enzymes of lipid metabolism, namely those enzymes that we deem "involved", as rendered from our studies on lipid composition and precursor label utilization. From our investigations thus far, those activities that will be investigated include stearol-CoA desaturase, diacylglycerol acyltransferase, enzymes of fatty acid synthesis (de novo and chain elongation), CDP-choline:1,2-diacylglycerol phosphocholinetransferase and phosphatidic acid phosphatase.

B. Significance

TPA, the most abudant and most active tumor-promoting agent of croton oil, has been used widely in the two-stage mouse skin carcinogenesis system (Boutwell, 1978) to study the mechanism of tumor promotion. Recent studies have shown that when tumor promoters are used singly they inhibit spontaneous and induced differentiation in murine erythroleukemia cells (Yamasaki et al., 1977; Fibach et al., 1979; Rovera et al., 1977), mouse neuroblastoma cells (Fibach et al., 1978), 3T3 fibroblasts (Diamond et al., 1977), avian myoblasts (Cohen et al., 1976), hamster epidermal cells (Sisskin and Barrett, 1981), and avian melanocytes (Payette et al., 1980). Conversely, studies with human myeloid leukemia cells (Huberman and Callaham, 1979; Rovera et al., 1979) and human melanoma cells (Huberman et al., 1979) have revealed a dramatic action of phorbol esters: one of induction rather than inhibition of cellular differentiation.

Although the action of TPA as an inducer of cell differentiation has been the object of considerable study, a variety of compounds, unrelated chemically, have been shown to induce morphological and biochemical alterations in numerous cell lines. DMSO induces erythroid differentiation in mouse erythroleukemia (Friend) cells; the morphological, biochemical, and immunological changes induced have been well characterized (Friend et al., 1972; Ross et al., 1972; Ikawa et al., 1973). The differentiation-inducing effect of DMSO has recently been demonstrated in human cells (Collins et al., 1978, 1979, 1980; Huberman et al., 1979) and retinoic acid has been shown to induce maximal differentiation (approx. 90%) in HL-60 cells at a concentration of 1 M, an amount much less than the concentration needed for DMSO to produce similar results (Breitman et al., 1980b). Thus TPA, DMSO, and retinoic acid all effectively induce

differentiation of the HL-60 line. Other compounds under study that likewise potentiate HL-60 cell differentiation include the antibiotic, tunicamycin (Nakayasu et al., 1980), the chemotherapeutic drug, actinomycin-D and compounds such as hypoxanthine and hexamethylene bisacetamide (Collins et al., 1980). Although these agents have not been extensively investigated, criteria for morphological and functional differentiation have been assessed in HL-60 cells following treatment. These studies suggest that HL-60 cells share common target sites for the induction by differentiation of these compounds.

Whereas a number of investigators have demonstrated that TPA addition to cell cultures causes myriad alterations in lipid metabolism, the effects of DMSO, retinoic acid and other inducers on lipid metabolism have not been characterized. The complexity of this area can be well appreciated for not only do tumor promoters induce differentiation in some cell lines and inhibit the differentiation process in others, preliminary studies have shown that inducer action on lipid metabolism is equally complex. In a recent study by Homma et al. (1980) dexamethasone-induced differentiation of mouse leukemia cells was shown to produce significant changes in cellular phospholipid composition. Friend leukemia cells induced to differentiate with DMSO, hexamethylene-bis-acetamide, or sodium butyrate displayed various lipid compositional modifications (Zwingelstein et al., 1980). It is well documented that TPA, DMSO, and retinoic acid induce terminal differentiation (granulocytic, macrophage) in HL-60 cells, based on mophological and biochemical criteria. Lipid metabolization studies utilizing a one-cell type -- multi-inducer system have not been carrid out. A study in this vein would firstly reveal if the various inducers share common actions on lipid metabolism and secondly serve to elucidate the role of lipids in the cellular differentiation process.

Rohrschneier and Boutwell (1973) and Balmain and Hecker (1974) examined the relationship between TPA-induced tumor formation in mouse epidermis and phospholipid metabolism and found that TPA stimulated the early synthesis of phosphatidylethanolamine and phosphatidylcholine. In bovine lymphocytes TPA causes an enhanced labeling with [methyl-^{3}H]choline of phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin (Wertz and Mueller, 1978). Likewise, HeLa cells incubated with low concentrations of TPA (10^{-9} to 10^{-8} M) show a rapid increase of choline incorporation into cellular phosphatidylcholine (Kinzel et al., 1979). Stimulation of phospholipid metabolism, before the markers of differentiation are expressed, and enhanced triacylglycerol synthesis have been shown to occur in HL-60 cells treated with TPA (Cabot et al., 1980). In addition to the general effect of TPA on phospholipid metabolism, TPA has also been shown to evoke other lipid-associated alterations in a variety of systems: these include modifications of lipid microviscosity in lymphoblastoid cells (Castagna et al., 1979), inhibition of adipose conversion in 3T3 fibroblasts (Diamond et al., 1977), stimulation of secretion of disaturated phosphatidylcholine from alveolar type II cells (Dobbs and Mason, 1978), enhancement of phospholipase activity and prostaglandin production (Levine and Hassid, 1977; Levine and Ohuchi, 1978), and alterations in ganglioside metabolism in human melanoma cells (Huberman et al., 1979).

As can be gathered, a considerable body of work has been accomplished demonstrating the action of TPA as a modulator of lipid metabolism; however, only recent evidence indicates that other inducers of HL-60 cellular differentiation likewise alter lipid metabolic processes. In work by Cooper et al. (1981) inducers of myeloid differentiation were shown to inhibit sterol and phospholipid synthesis, although this information was based solely on the

incorporation of radiolabeled acetate into cellular lipids. Acetate can serve as a precursor of glycerol, and be incorporated into fatty acids via de novo synthesis or by chain elongaion. Acyl group turnover in triacylglycerols and the different classes of phospholipids should also be considered when investigaing lipid metabolism via labeling techniques.

When comparing lipid metabolic aspects in undifferentiated and induced cells, it should be taken into account that the serum required to grow cultured cells is a rich source of fatty acids, which may enable the cells to sustain their required acyl group composition. For that reason we plan in turn to employ the serum-free culture system for HL-60 cells recently described by Breitman et al. (1980a), wherein they also demonstrate DMSO-induced differentiation to occur. A culture system such as this one can greatly simplify studies aimed at demonstrating lipid compositional modificaions inherent to the induction of differentiation and the determination of cellular liqid requirements that could potentiate the differentiation process.

Approaches used to investigate the functions of specific lipids in cellular biomembranes have centered on manipulating the fatty acid (Awad and Spector, 1976; Williams et al., 1974; Jenkin et al., 1970; Kitajima and Thompson, 1977), fatty alcohol (Cabot and Snyder, 1980), or phospholipid polar head group composition (Schroeder et al., 1976; Blank et al., 1975) of membrane phospholipids. Alterations accompanying the lipid modification of cellular membranes have been observed in lipid metabolism (Blank et al., 1975; Graff and Lands,1976), membrane-bound enzyme activities (Blank et al., 1979; Mavis and Vagelos, 1972; Malkiewiz-Wasowicz et al., 1977; Horwitz et al., 1974), cell growth (Cabot and Snyder, 1978; Horwitz et al., 1974), and membrane fluidity (King et al., 1977; Kimelberg and Papahadjopoulos, 1972). These findings are supportive of the important functional role of specific lipid components in

biological membranes of intact cells, and mark the significance of employing serum-free growth media and lipid supplementation to establish what lipid criteria exit for the induction of differentiation.

The evidence that retinoids as well as tumor promoters enhance deacylation of cellular phospholipids and stimulate prostaglandin production (Levine and Ohuchi, 1978) implies that essential fatty acids and prostaglandins play a role in cellular differentiation. In a recent study by Bonser et al. (1981), DMSO treatment of HL-60 cells was shown to result in the appearance of phospholipase and cyclooxygenase activities. Prostaglandin synthesis in a preadipocyte clonal line was shown to be maximal during cell proliferation and decrease dramaically at adipose conversion (Negrel and Ailhaud, 1981). Likewise, Ziboh et al. (1981) have shown a marked increase in the biosynthesis of PGF_2 in rapidly proliferating chloroleukemia cells, whereas no significant increase in PGF_2 occurred in quiescent hyperplastic marrow cells. In a study by Homma et al. (1980) differentiated mouse myeloid leukemia cells, when labeled with [^{14}C]arachidonic acid, were shown to synthesize and release prostaglandins, whereas untreated cells did not. These results strongly suggest that prostaglandins play a role in the macrophage-granulocytic differentiation process of myeloid leukemia cells. The levels of polyunsaturated fatty acids can be controled by growing cells in serum-free media thus limiting the availability of precursors for prostaglandin production. Do lipid criteria exist (i.e., essential fatty acid and prostaglandin levels) for differentiation to occur? In such an exprimental system, cells that are "essential fatty acid-poor" can be treated with low levels of arachidonic acid to test the effects of membrane modification on the potentiation of cellular differentiation.

A number of investigations have shown that various chemical supplements can

elicit human leukemia cell differentiation. The effect of these inducers provides morphological and biochemical markers for correlating cell differentiation with modifications of lipid metabolism. In this regard, a cell system in which markers for differentiation can be followed provides a unique model for studying lipid-related biochemical events during the differentiation process. Human myeloid leukemia cells are extremely useful for such studies, since the cells can be induced to differentiate by a select number of compounds. Lipids are important components of cellular membranes playing a role in fluidity regulation, enzyme activity, transport, and structure, and membranes are prime targets for TPA (Wenner et al., 1974; Lee and Weinstein, 1978; Sivak et al., 1972). The fact that TPA, DMSO, and retinoic acid induce like morphological and biochemical alterations in HL-60 cells, suggests that these cells share common target sites for the induction process. Although several investigations have suggested that the cell surface plays an important role in mechanisms association with differentiation (Voldavsky et al., 1976; Lotem and Sachs, 1975), there are few reports on changes of membrane components during cellular differentiation. The relationship of lipids, their compositional and metabolic aspects, to cellular differentiation has received little attention, mainly because of the lack of an appropriate model system wherein modifications of lipid metabolism can be correlated and their relevancy established in tandum with the appearance of differentiation markers.

C. Preliminary Studies

Our initial results that document dramatic alterations in lipid metabolism occurring in TPA-differentiated human myeloid leukemia cells have been published (Cabot et al., 1980). The data and description of these experiments can been seen in the attached reprint (Appendix I). Briefly, this work shows that TPA-induced differentiation of HL-60 cells is accompanied by a stimulation of phospholipid metabolism (before the markers of differentiation are expressed), enhanced incorporation of acetate into free fatty acids and neutral lipids, an increase in the amount of cellular triacylglycerols, and a selective incorporation of [1-^{14}C]hexadecanol into triacylglycerols and their ether-containing analog, alkyldiacylglycerols. Our more recent studies (Cabot and Welsh, Cancer Res., in press, Appendix I) have revealed that TPA treatment of HL-60 cells has a profound effect on fatty acid metabolism. Most notably we found that cells treated with TPA for 48 hr show a marked decrease in stearoyl-CoA desaturase activity. Also, acyl group analyses of lipids from untreated and TPA-treated cells showed that there were no differences in the fatty acid profiles of phospholipids, although marked differences occurred in the acyl group composition of triacylglycerols between control and differentiated cells. Thus it appears that HL-60 cells, differentiated by TPA, can maintain membrane fluidity parameters that are crucial to cellular function. Our studies on fatty acid metabolism show that TPA causes an enhancement of fatty acid labeling from [^{14}C]acetate; the mechanism of acetate incorporation into HL-60 undifferentiated and TPA-differentiated cells was not ascertained. The extent of de novo synthesis vs chain-elongation of preexisting fatty acids in induced and control cells is a point of investigation in the present proposal. In previous studies we have shown that the amount of cellular triacylglycerols is higher in TPA-differentiated cells

(3.2-fold increase over control with 8 x 10^{-10} M TPA). This modulation in neutral lipids was also verified in experiments using radiolabeled fatty acids (precursors of acyl moieties). In cells incubated with labeled stearic acid, the percent distribution of lipid radioactivity was highest in the phospholipids (controls), whereas in TPA-supplemented cells, substantially more label was associated with triacylglycerols.

We have recently initiated experiments utilizing DMSO and retinoic acid as induction agents. The cells were cultured in media containing serum and incubated in the presence of inducer for 6 days. Preliminary data show that the acyl group compositions of phospholipids are altered, compared with undifferentiated cells. This is interesting in light of the fact that DMSO has been shown to alter membrane fluidity in HL-60 cells. Although triacylglycerols are not major membrane components, they may serve as fatty acid stores. Differences in acyl group compositions of the triacylglycerols between control and induced cells was also shown to occur.

We have been successful in culturing HL-60 cells in serum-free media, according to the procedure described by Breitman et al. (1980). The cells are currently in their 26^{o} passage, and their fatty acid composition is relatively simple compared to cells grown in serum-rich media. The most marked change is the near absence of polyunsaturates; 16:0 + 16:1 + 18:1 account for >80% of the total acyl groups in phospholipids and triacylglycerols. Induction of differentiation in these cells by TPA, DMSO, and retinoic acid is currently being assessed by morphological and biochemical criteria. Early results are encouraging with promise that utilization of cells, with a comparitively simple lipid composition, will complement studies on lipid stasis and cellular differentiation. The action of TPA on HL-60 cells grown in serum-free media has not been hitherto assessed. We have conducted a morphological evaluation

of these cells (1.6×10^{-8} M TPA, 48 hr) and shown the population to consist primarily of macrophage-like cells (>70%).

I was trained in the area of lipid biochemistry, and more specifically, lipid enzymology. The pursuit of these disciplines has given me considerable experience in the fields of lipid metabolism, membrane biochemistry (membrane-enzyme-substrate interactions, membrane modification), and tissue culture. As a result of my present position, I feel well qualified to pursue these studies. The laboratory, headed by Dr. Fred Snyder, at the ORAU Medical and Health Sciences Division, has been a forerunner in the area of lipid metabolism. The facility is well equipped for carrying out the specialized analyses that are required to explore all facets of lipid biochemistry.

D. Methods of Procedure

1. Cell culture and induction of differentiation

a) Cell culture

The HL-60 cells (Collins et al., 1977), which we are using, were originally provided by Dr. R. C. Gallo, National Cancer Institute, Bethesda, Maryland, and we obtained the cells directly from Dr. E. Huberman, Biology Division, Oak Ridge National Laboratory. Cells will be grown in bacterial plastic petri dishes (No. 1007, Falcon) or cultured in 75 cm^2 Falcon flasks in an atmosphere of 5% CO_2 in air at 37°C using RPMI-1640 medium containing 20% fetal calf serum and supplemented with penicillin (100 units/ml) and steptomycin (100 g/ml). Serum-free cells will be grown in RPMI-1640 media containing insulin and transferrin as described by Breitman et al. (1980). In most experiments cells will be seeded at 2-2.5 x 10^6 cells/flask and treated with inducer the same day or 24 hr later.

b) Induction of differentiation

Culture medium will be used to make serial dilutions of a TPA-DMSO solution and added to the cells to provide the desired concentrations of TPA (10^{-10}-10^{-8} M). By this method DMSO does not exceed 0.01% in the growth flasks; this procedure has been routinely employed (Huberman and Callaham, 1979; Cabot et al. 1980). Cell incubations, in the presence of TPA, will be carried out for 48 hr.

Terminal differentiation induced by DMSO (Collins et al., 1978) will be

carried out by incubating cells for 6 days in media containing 1.25% DMSO, and in a like manner for cells grown in serum-free media (Breitman et al., 1980a). Retinoic acid (all-trans-retinoic acid) will be used to induce differentiation following the procedure outlined by Breitman et al. (1980b). Briefly, cells will be incubated with retinoic acid, 1×10^{-6} M, for 6 days. Retinoic acid will first be disolved in 95% ethanol and serial dilutions into growth media made such that the ethanol concentration is no higher than 0.1% in the culture flask. HL-60 cellular differentiation induced by actinomycin-D, hexamethylene bisacetamide (HMBA), and hypoxanthine, will be carried out according to established protocol (Collins et al., 1980). Cells will be incubated in the presence of actinomycin-D (5.0 ng/ml) for 6 days; a media change at 4 days, replaced with fresh media containing inducer, is required. HMBA (2 mM) and hypoxanthine (5 mM) induction occurs likewise over a 6-day incubation period.

c) Assessment of differentiation

Given that there is some degree of variability between cells grown in various laboratories, differentiation will be assessed periodically by performing differential counts on Wright stained preparations and by evaluation of established markers for myeloid differentiation. Morphological differentiation will be based on the percentage of myelocytes, metamyelocytes, and banded and segmented neutrophils vs. the control population that consists primarily of promyelocytes (90%). TPA-induced differentiation will be assessed by the percentage of macrophage-like cells resulting at the end of treatment. Other criteria will include cell attachment to the substratum, and cessation of growth. Biochemical markers for differentiation include NBT dye reduction (Segal, 1974; Collins et al., 1980), phagocytes of Candida abicans (Collins et

al., 1978), lysozyme release (Bigger, 1978), and specifically in the case of TPA, acid phosphatase activity for the macrophage differentiation produced (Vorbrodt et al., 1979).

d) Isolation of subcellular fractions

Plasma membranes will be isolated following a published protocol specific for HL-60 cells (Ip and Cooper, 1980). Nuclei will be isolated according to a procedure described for guinea pig polymorphonuclear leukocytes (Depierre and Karnovsky, 1973). This procedure employs the homogenization of cells in a medium that is slightly hypotonic followed by immediate restoration of isotonicity. By this method the integrity of subcellular organelles is maintained. The purity of the subcellular fractions will be established by assay of classical marker enzymes.

2. Lipid analyses

Cellular lipid composition will be compared in uninduced and induced leukemia cells with special regard to amount and types of phospholipids (choline-, ethanolamine-, serine-, and inositol-containing) neutral lipids (sterols, triacylglycerols, alkyldiacylglycerols, cholesterol esters) and acyl group compositions of the glycerolipids. Lipids will be extracted from control and differentiated cells by a modified method of Bligh and Dyer (1959) in which the methanol contains 2% glacial acetic acid. Both neutral and polar lipids can be resolved by thin-layer chromatography in a variety of solvent systems (Snyder, 1973). Neutral lipids will be separated on layers of Silica Gel G and phospholipids resolved on Silica Gel HR. A comparison of R_f values with

commercial standards will be used for identification of lipid classes. Quantitative analysis of thin-layer-resolved lipids is accomplished by H_2SO_4 charring of the chromatoplates (pre-run in diethylether, 100%) at 200°C followed by photodensitometry according to Privett et al. (1965). This method will be used to determine the contributing amounts of sterols, triacylglycerols, alkyldiacylglycerols, and cholesterol esters to the total lipid fraction. Total phospholipids are quantitated by measuring phospholipid Pi (Rouser et al., 1966), and in a like manner, Pi of the various phospholipid classes can be determined directly by analysis of H_2SO_4-charred lipids. Acyl group composition will be analyzed in phospholipids (total and individual classes) and triacylglycerols by first isolating lipids on preparative thin-layer chromatographic plates. Phospholipids, triacylglycerols, alkyldiacylglycerols, and cholesterol esters are facilely separated on chromatoplates developed in hexane/diethylether/acetic acid (80:20:1). Methyl esters will be prepared by refluxing the lipids in methanol containing 2% H_2SO_4 at 100°C (Ways et al., 1963) and analyzed by gas-liquid chromatography according to the methods described by Blank and Snyder (1970) and Blank et al. (1976). Quantitation will be based on weight·percent using a Hewlett-Packard recording integrator. Additionally, the positional specificity of the fatty acids in the major phospholipid classes will be determined by gas-liquid chromatography after treatment of the isolated phospholipids with phospholipase A_2 (Cabot and Snyder, 1978).

3. Lipid metabolic studies

a) Fatty acid metabolism

Fatty acid desaturase activity in undifferentiated and differentiated cells will be assayed using both whole cells (Blank et al., 1976) or cell-free homogenates (Oshino and Sato, 1972). Briefly, control cells and induced cultures will be incubated with [1-^{14}C]stearic acid for 1-2 hr. Methyl esters are then prepared from the lipid extracts of harvested, washed cells, and separated according to the degree of unsaturation on $AgNO_3$-impregnated thin-layer plates (Bandi and Mangold, 1969). Bands corresponding to monoenes will be visualized with iodine vapors and scraped for radioactive analysis by lipid scintilation spectrometry. The cell free system will consist of microsomes (0.2-1.0 mg protein) from control or induced cells, 0.1 M Tris-HCl buffer, pH 7.2, 30-70 M [1-^{14}C]steaoyl-CoA, and 0.4 mM NADPH. After a 5-min incubation at 30°C, the reaction is saponified, acidified, and methylated for thin-layer analysis. An alternate method of assay employs replacement of NADPH with an NADPH-generating system (1 mM $NADP^+$, 3 mM $MgSO_4$,10 mM glucose-6-P, excess glucose-6-P dehydrogenase). To evaluate the effects of differentiation on both fatty acid elongation and desauration, control and induced cells will be incubated with [1-^{14}C]palmitic acid for 1 hr and the metabolic products in the lipid extracts from cells analyzed by combined gas-liquid chromatography and collection of $^{14}CO_2$ as described by Blank et al. (1976).

Immature leukemic blast cells contain acetyl-CoA carboxylase and are capable of fatty acid synthesis de novo; however, human leukocytes lack acetyl-CoA carboxylase and incorporate acetate into fatty acids via chain elongation (Majerus and Lastra, 1967; Wakil, 1961). This interesting contrast between immature and differentiated cells can be employed as a marker for differentiation. For this reason, fatty acid synthesis, de novo, and by chain elongation will be assayed in control and differentiated prototypes. Intact cells will be incubated in regular media containing serum, or for short

labeling periods, washed and resuspended in serum-free media. [1-^{14}C]Acetate (10 Ci/flask) will be introduced and incubated for periods up to 24 hr. Aliquots of cellular total lipids are then hydrolyzed in 2 N methanolic KOH for 2 hr at 70°C and subjected to Schmidt degradation by the method of Brady et al. (1960). This technique, which removes the cabonyl carbon of fatty acids (C-1) is used to distinguish between de novo fatty acid synthesis and chain elongation. Radioactivity in the total fatty acid fraction will then be compared with the percentage evolved as $^{14}CO_2$ from the C-1 position. The overall capacity of undifferentiated and induced cells to synthesize fatty acids will be tested in cell-free extracts using the radioisotopic method described for determination of fatty acid synthase activity (Ahmad et al., 1979).

b) Acyl group modification of cellular lipids

The effects of altering membrane fluidity on the potentiation of induction will be tested on cells grown in media containing supplemented fatty acids. Cells grown in serum-containing or serum-free media can be manipulated to favor acyl group enrichment with a particular fatty acid (Wisnieski et al., 1973; Cabot and Snyder, 1978). Culturing cells in serum-free media drastically alters the acyl group composition of the glycerolipids (see Preliminary Studies). Because the effects of serum removal on cell differentiation ae not well understood, we will carry out our initial supplementation studies on cells grown in serum-containing media. Thus, any differences noted in the course of induction can be ascribed to the enrichment rather than a combination of acyl group enrichment and serum absence. We have considerable experience in the area of membrane modification; therefore, we anticipate that a 24-hr exposure to fatty acids (3-10 g/ml growth media) will be sufficient to accomplish

enrichment. Fatty acids (stearic and arachidonic) will be introduced as the NO^+ soaps and phospholipid acyl groups will be analyzed to determine the modifications achieved (Cabot and Snyder, 1978). Once a dose-response relationship and toxicity levels have been established, the enriched cells will be exposed to various concentrations of TPA, DMSO, or retinoic acid, and the time course for differentiation, based on morphological and biochemical criteria, will be determined. Breitman et al. (1980a) have shown that HL-60 cells grown in serum-free media can be induced to differentiate in the presence of DMSO; we have preliminary data that shows the same occurs with TPA. Therefore, we will employ cells grown in serum-free media as controls to correlate the inductive effects of DMSO and TPA on cells cultured in serum-free media containing either stearic or arachidonic acids. Radiolabeled stearic and arachidonic acids, incubated with cells for 24 hr, will be used as tracers to determine the distribution of fatty acid supplements in plasma membrane and nuclear fractions.

c) Metabolism of prostaglandin precursors

Our studies on prostaglandin metabolism in undifferentiated and differentiated cells will be limited to investigations centering on the assimilation of labeled prostaglandin precursors (linoleic and arachidonic acids), and the levels of phospholipase A_2 activity in cells. [1-^{14}C]Linoleic acid or [1-^{14}C]arachidonic acid will be incubated with control and induced cells to establish the time course of uptake and the distribution of radioactivity in cellular phospholipids (choline-, ethanolamine-, inositol-, and serine-containing glycerophospholipids) and triacylglycerols. The level of phospholipase A_2 activity will be assayed in control and differentiated cells

prelabeled with arachidonic acid according to the procedure described by Boner et al. (1981). Briefly, cell cultures will be incubated with [1-^{14}C]arachidonic acid (0.1 Ci/ml culture media). Washed labeled cells are then resuspended in assay buffer (50 mM Tris·HCl, pH 7.4, containing 100 mM NaCl, 1.4 mM $CaCl_2$, and 0.7 mM $MsCl_2$). The 1-ml cell suspensions (5 x 10^7 cells/ml) are then incubated with or without 10 M calcium ionophore A_{22187} for 5 min at 37°C. Solvent extractions of the reaction mixture will then be assayed for free arachidonate by thin-layer chromatography. Prelabeled cells will first be analyzed to determine the distribution of labeled arachidonate in each of the phospholipid classes. By this means we can correlate the appearance of free arachidonic acid with depletion of radioactivity in the particular phospholipid classes.

d) In vitro enzymic assays

Diacylglycerol acyltransferase will be assayed using microsomes (as enzyme source) isolated from control and differentiated cells according to established procedures (Coleman and Bell, 1976; Bell and Miller, 1976). Measurement of enzyme activity will also be carried out utilizing endogenous diacylglycerols. Briefly, microsomal fractions will be incubated with phospholipase C (Cabot and Gatt, 1977) to generate endogenous, membrane-bound diacylglycerols. These microsomes (0.2-0.4 mg protein) will then be incubated a 37°C in medium containing 50 mM NaF, 2.0 mM dithiothreitol, bovine serum albumin (5 mg/ml) and 0.1 mM [1-^{14}C]palmitoyl-CoA. Upon termination, the lipid will be extracted from the reaction mixture and radiolaeled triacylglycerols separated by thin-layer chromatography. Phosphatidic acid phosphatase activity will be measured by adding an exogenous dispersion of phosphatidic acid to the

measured by adding an exogenous dispersion of phosphatidic acid to the microsomal or soluble fraction of control or differentiated cell preparations (Lamb and Fallon, 1974; Caras and Shapiro, 1975). An alternate assay method will employ microsomal-bound [^{14}C]phosphatidate as substrate (Lamb and Fallon, 1974). Cholinephosphotransferase (CDP-choline:1,2-diacylglycerol phosphocholinetransferase) will be assayed by incorporation of phosphoryl-[Me-^{14}C]choline in phosphatidylcholine from CDP-[Me-^{14}C]choline. The procedure described by Weiss et al. (1958) and modified by Vance and Burke (1974) will be used in the assay of this enzyme.

4. Priorities

The priorities of our proposed work will be essentially according to the order outlined (pp. 1-9). However, preliminay data will be obtained through lipid compositional studies of cells treated with the various induction agents, and these results will direct future project decisions so that emphasis can be placed on mechanistic studies such as subcellular site of action, lipid enzymology, and the role of serum and essential fatty acids. We have a number of collaborative ties with scientists outside our group: cell biologists and toxicologists (Dr. E. Huberman, Argonne, Chicago), organic chemists (Dr. C. Piantadosi, University of North Carolina, Chapel Hill), mass spectroscopy (Dr. W. Rainey, Oak Ridge National Laboratory), cytogenetics (Dr. Gayle Littlefield, Oak Ridge Associated Universities). These scientists could be useful in solving some of the specialized problems that might arise.

Timetable

First year: We will concentrate our efforts documenting the lipid compositional changes associated with the induction of differentiation utilizing the various agents. Close scrutiny will be given to assessment of differentiation (morphological and biochemical criteria) so that the dynamics of lipid alterations can be correlated with the inducer and degree of differentiation. In turn, studies will be initiated using cells grown in serum-free media. Recruitment and training of a technician will be undertaken during the first 6 months.

Second year: All studies initiated during year 1 will be completed and data assessed to give direction to subsequent experimentation.

Third year: We will initiate and complete studies utilizing labeled lipid precursors. These data, together with the compositional studies will set the groundwork for enzymatic and mechanistic areas. As this work will closely complement some of our enzymatic studies, we will initiate aspects of fatty acid metabolism: desaturation, elongation, de novo synthesis.

Fourth year: Enzymatic studies will be continued to include examination of those activities outlined in Aims. We will initiate a specific study of arachidonic acid metabolism inclusive of phospholipase A_2 activity and prostaglandin production.

Fifth year: The effects of membrane modification on cellular differentiation will be studied by utilizing cells grown in serum-free media with or without supplemented fatty acids. As the specific experimental format for studies outlined may lend itself to studies aimed at the involvement of

plasma and nuclear membranes lipid and differentiation, we will, throughout the entire funding period, explore the effects of induction on lipid composition of subcellular fractions.

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E. FACILITIES AVAILABLE

Our laboratories are well equipped to conduct the experiments outlined in this proposal. We have recently added a new tissue culture facility containing both forced draft and water-jacketed incubators equipped with automatic CO_2 control, laminar flow hoods, a self-contained biohazard hood, and liquid nitrogen refrigerator. We have also recently established a laboratory specially designed for human cell culture. For lipid analyses we have a complete line of thin-layer chromatographic equipment, including a thin-layer zonal scraper, a spark chamber for chromatographic detection of ^{3}H and ^{14}C, and a photodensitometer designed for quantitative analysis. We also have the following equipment: preparative and analytical centrifuges with conventional rotors, an analytical Model E ultracentrifuge, liquid scintillation spectrometers, gas-liquid chromatographs with a variety of column packings, an automatic freeze dryer, Beckman DU and Acta C-III spectrometer, equipment for isolating proteins (electrophoresis apparatus, fraction collectors, columns), a Cahn electrobalance (Model 25), and high pressure liquid chromatographs. We also have access to electron microscopy.

'USE OF DOE FACILITIES AND DOE CONTRACT REQUIREMENTS'

This research grant application includes a segment of activity that would be performed in facilities of the U. S. Department of Energy and governed by an existing contract between Oak Ridge Associated Universities (ORAU) and the DOE. The DOE has reviewed this proposal and has concurred in ORAU conducting the described work in the DOE facilities made available for biomedical research, subject to payment to the DOE by ORAU from NIH funds of the applicable direct and indirect cost of the work (not including any charge for the use of DOE facilities) as determined by the provisions of the DOE's contract with ORAU.

It is belived that in large measure the requirements of the DOE contract parallel conditions that NIH ordinarily applies to its grants. In the event of differences between NIH grant terms and the DOE contract terms, ORAU is agreeable to meeting both to the extent that they are not in conflict, and to applying those most favorable to the United States Government where this is involved. If NIH is aware of problems that such an approach would produce or suggest, ORAU upon receipt of such advice would refer the matter to the DOE for direct resolution with NIH.

By way of general information, ORAU's contract with the DOE is a cost-type contract financed under a Government-fund account. The specific contract work is formulated in cooperation with the DOE and authorized within general guidelines in the contract. Contract terms include DOE responsibility for Government ownership and control of inventions, data, and other research products. Ownership of all equipment and facilities acquired by ORAU with DOE funds is vested in the U. S. Government at the time of acquisition. The contract also contains all the terms generally common to Government contracts of the type under which ORAU conducts research operations in Government-owned facilities.

F. Collaborative Arrangements: None

Privileged Commun. tion

C T. Myles C.
[redacted]

G. Principal Investigator Assurance

The undersigned agrees to accept responsibility for the scientific and technical conduct of the project and for provision of required progress reports if a grant is awarded as the result of this application.

_____

Principal Investigator

_____

Date