

SECTION I

DEPARTMENT OF  
HEALTH, EDUCATION, AND WELFARE  
PUBLIC HEALTH SERVICE

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TYPE	PROGRAM	NUMBER	718703
REVIEW GROUP		FORMERLY	
COUNCIL (Month, Year)		DATE RECEIVED	

GRANT APPLICATION

TO BE COMPLETED BY PRINCIPAL INVESTIGATOR (Items 1 through 7 and 14A)

1. TITLE OF PROPOSAL (Do not exceed 53 typewriter spaces)

FUNCTION OF CLL LYMPHOCYTES USING PURIFIED <sup>3</sup>H PHA

2. PRINCIPAL INVESTIGATOR

2A. NAME (Last, First, Initial)

Conard, Robert A.

2B. TITLE OF POSITION

Senior Scientist

2C. MAILING ADDRESS (Street, City, State, Zip Code)

Medical Department  
Brookhaven National Laboratory  
Upton, New York 11973

3. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)

FROM May 1, 1972 THROUGH April 30, 1977

4. TOTAL DIRECT COSTS REQUESTED FOR PERIOD IN ITEM 3

\$ 262,764

5. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH PERIOD

\$ 47,938

2D. DEGREE

B. S., M. D.

2E. SOCIAL SECURITY NO.

2F. TELEPHONE DATA

Area Code 516 TELEPHONE NUMBER AND EXTENSION 924-6262, Extension 2876

2G. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT (See Instructions)

Medical Research Center

2H. MAJOR SUBDIVISION (See Instructions)

Radiobiology Section

6. PERFORMANCE SITE(S) (See Instructions)

Medical Department  
Brookhaven National Laboratory  
Upton, New York 11973  
Congressional District No. 1

REPOSITORY \_\_\_\_\_

COLLECTION \_\_\_\_\_

BOX No. \_\_\_\_\_

FOLDER \_\_\_\_\_

7. INVENTION CERTIFICATION FOR RENEWAL APPLICATIONS ONLY. (See Instructions)

Have any inventions been conceived or first actually reduced to practice in the course of this project?  NO

Yes-previously reported.

Yes-not previously reported.

(Indicate as per Instructions.)

N/A

TO BE COMPLETED BY RESPONSIBLE ADMINISTRATIVE AUTHORITY (Items 8 through 13 and 14B)

8. APPLICANT ORGANIZATION(S) (See Instructions)

Associated Universities, Inc.  
Brookhaven National Laboratory  
Upton, New York 11973

10. TYPE OF ORGANIZATION (Check applicable item)

FEDERAL  STATE  LOCAL  OTHER (Specify)  
Non-profit-Private

11. NAME, TITLE, ADDRESS, AND TELEPHONE NUMBER OF OFFICIAL IN BUSINESS OFFICE WHO SHOULD ALSO BE NOTIFIED IF AN AWARD IS MADE

Lewis R. Burchill, Controller  
Associated Universities, Inc.  
Brookhaven National Laboratory  
Upton, New York 11973

Telephone Number 924-6262, Ext. 556

9. NAME, TITLE, AND TELEPHONE NUMBER OF OFFICIAL(S) SIGNING FOR APPLICANT ORGANIZATION(S)

N. Peter Rathvon, Secretary  
Associated Universities, Inc.  
Brookhaven National Laboratory  
Upton, New York 11973

Telephone Number (s) 924-6262 Ext. 2371

12. IDENTIFY ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT PURPOSES (See Instructions)

Brookhaven National Laboratory

13. PHS ACCOUNT NUMBER (Enter if known)

73-0887

14. CERTIFICATION AND ACCEPTANCE. We, the undersigned, certify that the statements herein are true and complete to the best of our knowledge and accept, as to any grant awarded, the obligation to comply with Public Health Service terms and conditions in effect at the time of the award.

SIGNATURES (Signatures required on original copy only. Use ink, "Pet" signatures not acceptable)	A. SIGNATURE OF PERSON NAMED IN ITEM 2A <i>Robert A. Conard</i>	DATE Aug. 31, 1971
	B. SIGNATURE(S) OF PERSON(S) NAMED IN ITEM 9	DATE 8362

4003785

## SECTION I

NOT FOR  
PUBLICATION  
OR  
PUBLICATION  
REFERENCEDEPARTMENT OF HEALTH, EDUCATION, AND WELFARE  
PUBLIC HEALTH SERVICE

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PROJECT NUMBER

## RESEARCH OBJECTIVES

NAME AND ADDRESS OF APPLICANT ORGANIZATION

Medical Department, Brookhaven National Laboratory, Upton, New York 11973

NAME, SOCIAL SECURITY NUMBER, OFFICIAL TITLE, AND DEPARTMENT OF ALL PROFESSIONAL PERSONNEL ENGAGED ON PROJECT,  
BEGINNING WITH PRINCIPAL INVESTIGATORRobert A. Conard, , Senior Scientist, Medical Department.  
Yang H. Oh, 497-42-7155, Research Collaborator, Medical Department.

TITLE OF PROJECT

FUNCTION OF CLL LYMPHOCYTES USING PURIFIED <sup>3</sup>H-PHAUSE THIS SPACE TO ABSTRACT YOUR PROPOSED RESEARCH. OUTLINE OBJECTIVES AND METHODS. UNDERSCORE THE KEY WORDS  
(NOT TO EXCEED 10) IN YOUR ABSTRACT.

Functional defects in the lymphocytes may occur in chronic lymphocytic leukemia (CLL) and certain other disorders associated with impaired immunocompetence. Phytohemagglutinin (PHA), a potent mitogenic protein extracted from kidney beans, causes widespread blastogenesis and proliferation of cultured lymphocytes from normal individuals; but this response is reduced in lymphocytes from persons having disorders associated with impairment of cellularly mediated immune mechanisms, such as CLL, Hodgkin's disease, sarcoidosis, and even aging. Although PHA has been used in numerous studies, the intracellular mechanisms of its action remain obscure. Our prime objective is to elucidate these mechanisms in normal lymphocytes and in lymphocytes from people with the above disorders, in order to clarify whether these disorders involve basic cellular defects, and possibly to indicate therapeutic approaches. We are isolating purified proteins with a high degree of mitogenicity from PHA by physico-chemical techniques and labeling them with tritium and fluorescent tags. Studies using the tagged mitogens (some already in progress) include localization of mitogens within membranes and cells by autoradiography (bright field and electron microscopy) and subcellular fractionation; and enzymatic and metabolic studies of mechanisms of action at the organelle level, including nuclear histone modification. Long-term objectives are to identify the biologically active sites of the mitogenic molecules and determine their structural conformation; and to make use of the mitogen in the search for an intralymphocytic virus in CLL.

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DETAILED BUDGET FOR FIRST 12-MONTH PERIOD

FROM  
May 1, 1972

THROUGH  
April 30, 1973

DESCRIPTION (Itemize)		HRS PER WEEK	AMOUNT REQUESTED (Omit cents)		
PERSONNEL	SALARY		FRINGE BENEFITS	TOTAL	
NAME	TITLE OF POSITION				
<b>(a) Stipulated salaries</b>					
Yang H. Oh, Ph.D.	Research Collaborator	40	17,100	2,736	19,836
Michael Makar, B.Sc.	Technical Assistant	40	9,750	1,560	11,310
Technical Assistant	Technical Assistant	40	8,700	1,392	10,092
		<b>Total</b>	<b>35,550</b>	<b>5,688</b>	<b>41,238</b>
<b>(b) Non-stipulated salaries</b>					
Robert A. Conard, M.D.	Senior Scientist	20	-	-	-
William A. Scott	Technical Assistant	20	-	-	-
CONSULTANT COSTS <u>None</u>					
EQUIPMENT <u>Refrigerated Fraction Collector \$3,000</u>					
SUPPLIES <u>Chemicals, special and routine glassware, ion-exchange absorbents, supplies for electrophoresis, UV, IR, CD, chromatography, culture media</u>					
					1,000
<u>Animals (Rodents)</u>					500
TRAVEL	DOMESTIC	<u>Two trips</u>			800
	FOREIGN				-
PATIENT COSTS (See instructions)					
ALTERATIONS AND RENOVATIONS					
OTHER EXPENSES (Itemize)					
		Publication costs	150		
		Books	100		
		Photography	150		
		Instrument repair & maintenance	1,000	1,400	
TOTAL DIRECT COST (Enter on Page 1, Item 5)					47,938

INDIRECT COST  
(See Instructions)

\_\_\_\_\_ % SSW\*  
35 % TDC\*

DATE OF DHEW AGREEMENT:

WAIVED  
 UNDER NEGOTIATION WITH:

DHEW-DGAP AGR. 12/16/70

\*IF THIS IS A SPECIAL RATE (e.g. off-site), SO INDICATE. (Excluding equipment)

**BUDGET ESTIMATES FOR A . . . YEARS OF SUPPORT REQUESTED . . . FROM PUBLIC HEALTH SERVICE  
DIRECT COSTS ONLY (Omit Cents)**

DESCRIPTION	1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED <i>(This application only)</i>					
		2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS	41,238	43,713	46,335	49,116	52,062		
CONSULTANT COSTS <i>(Include fees, travel, etc.)</i>	-	-	-	-	-		
EQUIPMENT	3,000	2,500	2,800	1,500	-		
SUPPLIES	1,500	1,600	1,700	1,800	1,900		
TRAVEL	DOMESTIC	800	800	800	800	800	
	FOREIGN	-	-	-	-	-	
PATIENT COSTS	-	-	-	-	-		
ALTERATIONS AND RENOVATIONS	-	-	-	-	-		
OTHER EXPENSES	1,400	1,500	1,600	1,700	1,800		
TOTAL DIRECT COSTS	47,938	50,113	53,235	54,916	56,562		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD <i>(Enter on Page 1, Item 4)</i> →					\$ 262,764		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

## SECTION II - PRIVILEGED COMMUNICATION

## BIOGRAPHICAL SKETCHES

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Robert A. Conard	Senior Scientist	
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
Jacksonville, Florida, U. S. A.	U. S. Citizen	<input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

## EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of South Carolina	B. S.	1936	Biology
Medical College of South Carolina	M. D.	1941	Medicine
University of California	No Degree	1947	Nuclear Medicine

HONORS Appointed Rear Admiral (MC) USNR-1945. Military honors not listed here. Visiting Professor of Pathology, State University of N.Y. at Stony Brook. Committee membership: NAS, aging (ad hoc committee), Radiobiological Advisory Panel, Space Science Board, Editorial Board "Radiation Research."

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Biomedical Research	Principal Investigator

RESEARCH SUPPORT (See instructions) Associated Universities, Inc., Contract No. AT(30-1)-16 with the U. S. Atomic Energy Commission.

I participate in an NIH post-doctorate training program in Gerontology (HD 00199-05). A post-doctorate and a technician work with me on aging problems at the cellular level. Funding for the current grant year for this problem is \$22,160.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. (Do not exceed 3 pages for each individual.)

1956- Senior Scientist, Medical Research Center, Brookhaven National Laboratory: Bio-medical research. Studies of effects of phytohemagglutinin on human lymphocytes in culture; purification and tagging; localization and mechanisms of action; use in studies on aging and on chronic lymphocytic leukemia. In charge of a medical team carrying out annual medical examinations on Marshallese people accidentally exposed to radioactive fallout in 1954. Studies of this population have furnished important information on radiation effects on human beings, particularly late effects such as thyroid abnormalities and growth retardation in children.

1950-56: Naval Medical Research Institute, Bethesda, Maryland: Radiobiological studies of animals, particularly effects of radiation on physiology of the gastrointestinal tract of rodents; beginning participation in human radiation studies of exposed Marshallese people.

1949- Argonne National Laboratory: Training in biological research (radiation effects on animals).

1948- University of California: Course in medical physics.

1941-48: U. S. Navy: Physician on ships and in naval hospitals; beginning in 1946, radiologic safety program associated with atomic bomb testing; later, Projects Officer for Medical Research at the Radiobiological Defense Laboratory, San Francisco, California.

BIBLIOGRAPHY - Robert A. Conard

Not listed here are numerous publications, over the past 15 years, on medical studies of the Marshallese people exposed to fallout radiation, and, over the past 20 years on effects of radiation in animals, particularly on gastrointestinal physiology, bone growth, etc. The papers listed, including some in preparation, concern current research more directly relevant to this proposal.

1. Reduction in size of succeeding generations of daughter cells in PHA-stimulated human lymphocytes. In preparation.
2. Autoradiographic studies of leukocytes cultured with tritiated bean extract. R. A. Conard, Nature 214: 709-10, 1967.
3. Preparation and purification of tritiated phytohemagglutinin and studies of cellular localization in human leukocyte cultures. R. A. Conard and C. F. Demoise, Blood 35: 44-55, 1970.
4. Quantitative study of radiation effects in phytohemagglutinin-stimulated leukocyte cultures. R. A. Conard, Int. J. Radiat. Biol. 16(2): 157-65, 1969.
5. Some properties of mitogenic components isolated from phytohemagglutinin by a preparative gel electrophoresis. Y. H. Oh and R. A. Conard, Arch. Biochem. Biophys., in press, 1971.
6. Tritiation of mitogenic glycoproteins for studies in cultured lymphocytes. Y. H. Oh and R. A. Conard, Abstract, 162nd ACS Meeting, 1971; submitted to Nature.
7. Effect of aging on transformation and histone acetylation of human lymphocytes stimulated by phytohemagglutinin. Y. H. Oh and R. A. Conard, submitted to Science.
8. Circular dichroism studies on two mitogenic components from phytohemagglutinin. Y. H. Oh and R. A. Conard, submitted to Biochemistry.
9. Aging studies in a Marshallese population exposed to radioactive fallout in 1954. R. A. Conard, A. Lowrey, M. Eicher, K. Thompson, and W. A. Scott. In Proceedings of a Colloquium on Radiation and Ageing, Semmering, Austria, June 1966, Taylor and Francis, Ltd., London, England.
10. Chromosome studies on Marshallese Islanders exposed to fallout radiation. H. Lisco and R. A. Conard, Science 157: 445-7, 1967.
11. Immunohematological studies of Marshall Islanders sixteen years after fallout radiation exposure. R. A. Conard, C. F. Demoise, W. A. Scott, and M. Makar, J. Gerontol. 26: 28-36, 1971.

## BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Yang H. Oh	Research Collaborator	
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
Pusan, Korea	Korean, permanent residence	<input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Central Mo. State College, Warrensburg	B. S.	1958	Chemistry
University of Missouri, Columbia	M.S, Ph.D.	1964	Biochemistry
Harvard Medical School, Boston	Postdoctoral	1966-1969	Biochemistry

HONORS The Society of Sigma Xi (1964), NIH postdoctoral fellowship (1967-1969) 7 F2 AM-25 929-01A1 (m)

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Nuclear Proteins and Mitogens	Associate Investigator, 40 hours per week

RESEARCH SUPPORT (See instructions)

NIH Gerontology Training Grant No. HD 00199-05

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

1969- Research Collaborator, Medical Department, Brookhaven National Laboratory. Isolation, characterization, and radioactive labeling studies on phytomitogens and phytohemagglutinins. Modification of nuclear histones with aging. Work performed with Dr. Robert A. Conard, Brookhaven National Laboratory.

1968-69: Associate in Biological Chemistry, Harvard Medical School, Boston, Mass.

1966-68: Research Fellow in Biological Chemistry, Harvard Medical School, Boston, Mass. Fractionation, characterization, and physical-chemical structure (amino acid sequences) studies on nuclear histones. Conformation of proteins and nucleic acids in solution; optical rotatory dispersion, circular dichroism, UV-IR absorption spectroscopy. X-ray diffraction. Work performed with Dr. Elkan R. Blout, Harvard Medical School.

1965-66: Research Associate in Biochemistry, State University of New York at Buffalo, Buffalo, New York. Fractionation and characterization of human plasma proteins and binding studies of plasma protein fractions to small molecules. Work performed with Drs. Richard J. Winzler and Benjamin E. Sanders, State University of New York at Buffalo.

1964-65: Instructor in Biochemistry, University of Missouri, Columbia, Missouri. Fractionation, characterization, structure, and interaction studies on wheat proteins and on caseins. Work performed with Dr. Charles W. Gehrke, University of Missouri.

1958-60: Instructor in Chemistry, Korean Military Academy, Seoul, Korea.

BIBLIOGRAPHY - Yang H. Oh

1. Some properties of mitogenic components isolated from phytohemagglutinin by a preparative gel electrophoresis. Y. H. Oh and R. A. Conard, Arch. Biochem. Biophys., in press, 1971.
2. Tritiation of mitogenic glycoproteins for studies in cultured lymphocytes. Y. H. Oh and R. A. Conard, Abstract, 162nd ACS Meeting, 1971; submitted to Nature.
3. Effect of aging on transformation and histone acetylation of human lymphocytes stimulated by phytohemagglutinin. Y. H. Oh and R. A. Conard, submitted to Science.
4. Circular dichroism studies on two mitogenic components from phytohemagglutinin. Y. H. Oh and R. A. Conard, submitted to Biochemistry.
5. Spectroscopic studies of five purified histones from calf thymus. Y. H. Oh, J. Biol. Chem. 245: 6404, 1970.
6. Binding of biologically active amines to plasma protein fractions. Y. H. Oh, E. Leitch, S. M. Small, R. J. Winzler, and B. E. Sanders, Biochem. Pharm. 16: 849, 1967.
7. Improved chromatographic fractionation and characterization of human plasma proteins. Y. H. Oh and B. E. Sanders, Anal. Biochem. 15: 232, 1966.
8. Characterization of a homogeneous gamma-A-globulin obtained from Cohn's plasma fraction III. (Abstract). Y. H. Oh, B. E. Sanders, and R. J. Winzler, Fed. Proc. 25: 2623, 1965.
9. Isolation and characterization of two major gamma-G-globulins from normal human plasma. Y. H. Oh and B. E. Sanders, Life Sci. 5: 827, 1966.
10. Chromatographic subfractionation and characterization of Cohn's fraction III from human plasma. Y. H. Oh, J. W. Nawrocki, W. E. Leitch, and B. E. Sanders, Anal. Biochem. 16: 220, 1966.
11. Physico-chemical properties of S-zone and base proteins in gluten. Y. H. Oh, B. E. Sanders, and C. W. Gehrke, Can. J. Biochem. 44: 917, 1966.
12. Stepwise elution chromatographic fractionation on CM-cellulose of S-zone proteins from gluten. Y. H. Oh and C. W. Gehrke, Anal. Biochem. 10: 148, 1965.
13. Stepwise elution chromatographic isolation on CM-cellulose of A<sub>s</sub>, G<sub>s</sub>, base, and A<sub>f</sub> proteins from wheat albumins and globulins. Y. H. Oh and C. W. Gehrke, Anal. Biochem. 10: 409, 1965.
14. Studies on gamma-A-globulins and alpha-2-macroglobulins. B. E. Sanders and Y. H. Oh, Can. J. Biochem. 46: 211, 1968.

BIBLIOGRAPHY-Yang H. Oh (Cont'd.)

15. Additional studies on plasma proteins obtained from schizophrenics and controls. B. E. Sanders, S. M. Small, W. J. Ayers, Y. H. Oh, and S. Axelrod, Trans. N. Y. Acad. Sci. 28: 22, 1965.
16. Chemical fractionation and starch gel-urea electrophoretic characterization of albumins, globulins, gliadins and glutenins in soft wheat. C. W. Gehrke, Y. H. Oh, and C. W. Freeark, Anal. Biochem. 7: 439, 1964.
17. Carboxymethyl cellulose column chromatographic fractionation and starch gel-urea electrophoretic characterization of soft wheat proteins. C. W. Gehrke, Y. H. Oh, and C. W. Freeark, Anal. Biochem. 8: 434, 1964.
18. Isolation of electrophoretically pure beta-caseins. C. W. Gehrke, C. W. Freeark, Y. H. Oh, and P. Chun, Anal. Biochem. 9: 423, 1964.
19. Isolation and polyacrylamide gel-urea electrophoretic characterization of alpha-s1-casein. C. W. Gehrke, P. Chun, and Y. H. Oh, Separation Sci. 1(4): 431, 1966.
20. Isolation and characterization of two mitogenic proteins from Phaseolus vulgaris. Y. H. Oh and R. A. Conard, Abstract Biol. 172, 160th ACS Meeting, 1970.
21. Isolation and characterization of a basic protein component from calf-thymus histones. Y. H. Oh and E. R. Blout, Abstract 173C, 154th ACS Meeting, 1967.
22. Spectroscopic studies of five purified histones from calf thymus. Y. H. Oh and E. R. Blout, Abstract Biol. 251, 158th ACS Meeting, 1969.

SECTION II

## RESEARCH PLAN:

A. Introduction1. Objective

The over-all objective of this project is first to elucidate the intracellular mechanisms induced by mitogens in cultured human lymphocytes from normal individuals and then to search for defects in lymphocytes from patients with diseases characterized by deficiency of cellular immunity, particularly chronic lymphocytic leukemia (CLL). Mitogen(s) will be isolated by a physico-chemical procedure in pure form from phytohemagglutinin (PHA), a potent mitogenic agent obtained from extracts of kidney beans. The isolated molecules will be labeled with tritium and fluorescent tags for study of intracellular localization and mechanism of action. Identification and characterization of the mitogenic determinants of the molecule will be an important longer-range objective of this study. Another objective is to make use of the virus-enhancing action of the mitogens in the search for a possible virus that might be associated with lymphocytic leukemias.

2, 3. Background and Rationale

The small lymphocyte is emerging as the important cell involved in maintaining immunological integrity of an individual. The literature concerning the role of lymphocytes in immune mechanisms has expanded tremendously in recent years. Thymidine labeling studies have shown that lymphocytes are of two kinds based on life-span: a longer-lived lymphocyte which is processed by the thymus and controls cellularly mediated immunity, and a shorter-lived lymphocyte derived directly from the bone marrow and not processed in the thymus which is involved in humoral immunity (antibody production).<sup>1</sup> The thymus-dependent lymphocytes recirculate through the peripheral blood and lymphatics, and the antibody-producing lymphocytes appear to be more localized in follicular tissues. Most of the small lymphocytes found in the peripheral blood are of the thymus-dependent type. The main function of the thymus-dependent lymphocytes is believed to be immunologic surveillance, i.e., recognition of and interaction against foreign antigens such as bacteria and viruses, transplantation antigens (histocompatibility antigens), and altered "self" proteins (autoimmune processes), and the recognition and destruction of cells with somatic mutations including malignant ones. These lymphocytes are capable of developing "memory" for specific antigens and are responsible for anamnestic reactions (augmented responses following a repeated challenge with the same antigen).<sup>2</sup> It is largely these lymphocytes that undergo *in vitro* transformation and proliferation when stimulated by mitogens of PHA in peripheral blood cultures.

Loss of cellularly mediated immunity is usually associated with reduction or impaired function of thymus-dependent lymphocytes. Also, reduced surveillance associated with diminished cellular immunity may be associated with the development of autoimmune diseases. Reduced and delayed blastogenesis of lymphocytes in peripheral blood culture in response to antigenic stimulation is believed to reflect the state of immunocompetence of the individual.<sup>3</sup> The reduced blastogenic response of cultured lymphocytes from patients with diseases characterized by reduced cellular immunity is significant, and we are interested in investigating possible altered intracellular function of lymphocytes that might account for it. The purified labeled mitogen from PHA will be a valuable tool in this study.

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The discovery a decade ago<sup>4</sup> that extracts from beans (PHA) cause widespread blastogenesis and cell division in lymphocytes in culture led to numerous investigations of the nature and action of this agent. The action of PHA as well as of some other plant antigens (such as those from jack bean, wisteria, and pokeweed) and certain other agents (streptolysin, staphylococcal extracts, antileukocyte antibodies, etc.) appears to be nonspecific in comparison with that of more specific agents such as tuberculin, tetanus toxin, typhoid and pertussis vaccines, etc. The latter agents cause much less blastogenesis, and the response occurs later than in cultures stimulated by "nonspecific" agents. Another difference is that prior "processing" of the antigen by macrophages appears to be necessary with specific antigens but not with nonspecific ones.

Some of the major changes reported to occur in PHA-stimulated lymphocytes are as follows: There is early leukoagglutination and erythroagglutination<sup>6</sup>, and in two days lymphocytes enlarge markedly; heterochromatin changes to euchromatin, and nucleoli become prominent; and the cytoplasm shows vacularization and basophilic staining. Within a few hours after addition of PHA increases occur in RNA and protein synthesis,<sup>7</sup> in ATP, oxidative enzymes, and glycolysis,<sup>8,9</sup> and in numbers of organelles such as mitochondria<sup>10</sup> and lysosomes.<sup>11</sup> These changes occur before the start of DNA synthesis. DNA synthesis begins at about 24 to 36 hours, and cell division at about 42 hours.<sup>12</sup> Formation of a small amount of gamma globulin by these cells has been reported by some investigators, though antibody formation does not appear to be prominent.<sup>13</sup> (Endoplasmic reticulum is sparse compared with the amount in plasma cells.<sup>14</sup>) These processes of transformation of the lymphocyte are further complicated by production and excretion of a number of effector molecules, such as transfer factor, macrophage inhibitor factor, lymphotoxin, mitogenic factor, and several others. The complexity of these changes testifies to the amount of research needed before the intracellular mechanisms are made clear.

Several attempts have been made to use PHA in vivo, and it appears to produce an immunosuppressive effect; however, in vivo use is hampered by the development of hypersensitivity to the numerous proteins in the crude form of PHA used. We hope to overcome this difficulty by isolating for use the mitogenic molecules from PHA in pure form.

During our earlier studies we perfected rapid techniques for electronic counting and sizing of cultured lymphocytes that allowed us to determine the amount of lymphocyte transformation in a large number of cultures in a short time, circumventing the more laborious measurement by microscope or tritiated thymidine uptake assays. These improved techniques greatly facilitated some of our basic studies on the kinetics of lymphocytes in cultures and permitted rapid assay of numerous fractions for mitogenicity, which was particularly important during our physical-chemical isolation procedures, described below.

In studies reported in the literature, the PHA used is a crude or partially purified extract and contains several biologically active components such as leukoagglutinating and erythroagglutinating activities. Several investigators<sup>13,15</sup> have shown the erythroagglutinins to be distinct from the other active principles and were able to remove them by adsorption to red blood cells. Subsequently, Rivera and Mueller<sup>16</sup> observed that the leukoagglutinating and RNA- and DNA-stimulating activities could be partially separated by protein purification techniques. Numerous other investigators<sup>16-21</sup> were unable to isolate any one of these activities not grossly contaminated with others. The mitogenic substances have been characterized by these investigators as glycoprotein in nature. Goldberg et al.<sup>22</sup> reported that a preparation obtained from PHA contained the RNA- and DNA-synthesis-stimulating activities free of the erythro- and leukoagglutinins and virtually free

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of protein and carbohydrate, but more recently Goldberg<sup>23</sup> has informed us that he now believes the mitogenic active principle to be protein in nature.

For the past three years we have been attempting to isolate the mitogenic principle from bean extracts, and we recently reported studies using a relatively pure protein.<sup>24</sup> Tagging with tritium was accomplished by growing the bean plants in tritiated nutrient media. With this tagged mitogen, autoradiographic and subcellular fractionation studies of transformed lymphocytes showed cytoplasmic, probably mitochondrial, localization of the label. However, the specific radioactivity of our mitogenic protein was too low (0.5  $\mu$ Ci/mg) to permit specific organelle visualization by autoradiography of electron microscopic sections. Reports of intracellular localization by use of conjugated<sup>25,26</sup> or labeled PHA<sup>27-29</sup> or conjugated antisera<sup>30-31</sup> have revealed rapid entrance of the label into the cytoplasm and in some cases into the nucleus. In our earlier studies<sup>28</sup>, we noted nuclear localization (in addition to cytoplasmic) of the tagged material which we believe was due to impurity of the PHA used. True intracellular localization of a labeled specific mitogenic molecule in the studies referred to above is questionable, since PHA of varying degrees of purity was used. In current work, we have achieved further isolation in essentially pure form of two distinct protein components (mitogens A and B) by a preparative gel electrophoresis technique;<sup>32</sup> these show greatly increased mitogenicity and almost complete lack of agglutinating activity. Greatly increased specific radioactivity (7 to 10  $\mu$ Ci/mg) of these proteins has been achieved by direct incorporation of tritium tagging by acetylation procedures.<sup>33</sup> Fluorescent tagging also is being attempted. Our preliminary results show predominantly cytoplasmic localization of the mitogenic label. Use of these purified and labeled mitogens should permit us to determine precise intracellular localization of the mitogen at the organelle level by autoradiographic procedures (bright field and electron microscopy) and radioactive assay of subcellular fractions. The purity of the isolated mitogens is essential for the more complex biochemical studies of intracellular mechanisms of action to be described in detail below. Other biochemical studies will include correlation of nuclear histone modifications with other manifestations of PHA mitogen stimulation of lymphocytes. Histones are believed to be associated with DNA in control of gene activation (derepression). Allfrey et al.<sup>34</sup> have demonstrated an increase in acetylation of "arginine-rich" histones of lymphocytes as early as 15 minutes after exposure to PHA. The technique previously used to study nuclear histones of calf thymus cells by Dr. Oh (one of our group) will be applied to this work.<sup>(35)</sup>

Loss of immunologic competence is a prominent feature of many lymphoproliferative diseases. With paraglobulinemias humoral antibody producing lymphocytes are defective or deficient. Impaired cellularly mediated immunity (thymus-dependent lymphocytes) is a feature of many conditions such as infectious mononucleosis, DiGeorge's syndrome, CLL, Hodgkin's disease, sarcoidosis, and other malignant states.<sup>5</sup>

Loss of immunological competence is also noted in aging. In a Marshall Island population we have found a definite increase in both gamma globulins and specific immunoglobulins as a function of age<sup>36</sup>. (But a Marshallese population that had been exposed to radioactive fallout showed depression of gamma globulins, particularly IgG and IgA.) Lymphocyte levels and response of cultured lymphocytes to PHA stimulation were reduced as a function of age. Preliminary studies also revealed decreasing acetylation of nuclear histones in cultured lymphocytes due to PHA stimulation which paralleled the decreasing response to PHA with age.<sup>37</sup>

CLL is a notable example of a disease which exhibits cellular anergy<sup>5,38</sup>. We intend to concentrate on this disease in our investigations of intracellular lymphocytic

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functions. In CLL the lymphocyte exhibits reduced and impaired RNA synthesis, impairment of glycogen metabolism, and low reactivity to PHA and other antigens;<sup>38, 39</sup> and in preliminary experiments we have also noted reduced and delayed response to the stimulatory action of PHA, and impaired nuclear histone acetylation. The degree of reduced blastogenesis appears to be correlated with the severity of the disease.<sup>38-41</sup> Elves<sup>40</sup> has suggested that there are two populations of lymphocytes in CLL, one normal which can respond to PHA stimulation and the other abnormal or leukemic. It is generally believed that the CLL lymphocyte is defective. The techniques we are developing should permit us to investigate the possible defect, and also to determine the effects on the lymphocyte function in CLL of spontaneous remission and exacerbation of the disease and of immunosuppression by radiation, drugs, and hormones.

The recent finding that virus appears to be enhanced in lymphocytes stimulated by PHA makes it possible to demonstrate the presence of virus particles in lymphocytes in herpes simplex,<sup>42</sup> vesicular stomatitis,<sup>43</sup> and bovine leukemias and lymphomas.<sup>44</sup> We plan to search for such particles in PHA-stimulated CLL lymphocytes by electron microscopic techniques.

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B. Specific Aims

In order to achieve our ultimate objective, outlined in A-1, i.e., to characterize possible functional defects in lymphocytes of CLL and other disorders exhibiting deficiency of immunocompetence, the following specific investigations should be carried out in the order given:

1. Physico-chemical isolation of the mitogenic molecules from PHA.
2. Determination of the conformation and identity of the biologically active sites of the mitogenic molecules. (This is a long-term study and will run in parallel with most of the others.)
3. Radioactive labeling of the mitogenic principle with tritium by direct labeling of the purified component ( $^3\text{H}$ -acetylation), and fluorescent tagging.
4. Intralymphocytic localization of the site of action of the labeled mitogens as a function of time by autoradiography (bright field and electron microscopy) and radiological assays of subcellular fractions.
5. Investigation of the mechanism of action of the mitogens by a variety of studies: (a) interaction (complexing) of the mitogen with various cellular elements; (b) correlation of the site of action of the mitogen with changes in RNA, DNA, and protein synthesis by biochemical and histochemical studies; (c) nuclear histone derepression (acetylation studies) as a function of mitogenic action; (d) metabolic studies of separated, purified organelle fractions, particularly mitochondria, by direct stimulation of such separated organelles with the mitogen.
6. Use of the enhancing effect of PHA mitogens to search for a virus in lymphocytes of CLL by electron microscopic techniques.

C. Methods of Procedure

Experiments are outlined below in the expected chronological order. The results of some preliminary work are indicated.

We have already perfected standard techniques for culture of human lymphocytes and for rapid assay of cultures for mitogen-induced transformation of lymphocytes. For assay, the culture is treated at the time of harvest with a proteolytic enzyme (pronase) to remove debris followed by cetrimid to strip away the cytoplasm by the method of Stuart and Ingram, and the bare nuclei are counted and sized by electronic means (Coulter). In some cases the data are fed into a computer and a complete sizing curve for all the cells in the culture is obtained. This method avoids the tedious microscopic differential cell counting or  $^3\text{H}$ -thymidine assay and allows rapid determination of percentage transformation of lymphocytes based on nuclear size. In the mitogen isolation procedures described below, this technique is of great value in permitting mitogenic assay in large numbers of column fractions at once. It has also been invaluable in basic studies on the kinetics of lymphocytes in culture, not directly a part of this project.

1. Isolation of the Mitogenic Molecules from PHA by Physical-Chemical Techniques.

We have reported preliminary studies with a partially purified mitogenic protein isolated, from kidney beans grown in tritiated nutrient media, by ammonium

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sulfate precipitation and column chromatography. Further progress is being made on the isolation of two mitogenic components from PHA by a preparative gel electrophoresis with urea-acrylamide. These two components have been tested for purity by analytical gel electrophoresis, analytical ultracentrifugation, and determinations of chemical compositions and of N-terminal amino acids.

## 2. Conformation and Identity of the Active Sites of the Mitogenic Molecules.

Sequential analysis for primary structure of the purified mitogens will be performed by the usual techniques such as use of the amino acid analyzer, Edman's step-wise degradation, peptide mapping (finger-printing), and enzymatic degradation. Techniques for specific modification of the mitogens will permit studies on the number of active sites, primary structure (active amino acid residues), and relationships between structure and function. Spectroscopic studies of the conformations of the mitogens in solution will include optical rotatory dispersion and circular dichroism as well as infrared and nuclear magnetic resonance spectroscopy. The conformational behavior will be related to possible structural roles of the mitogens in the transformation of lymphocytes.

## 3. Tagging of the Mitogen Molecules.

Preliminary radioactive labeling of PHA obtained from bean plants was accomplished by growing the plants in tritiated nutrient media (7 to 12 Ci  $^3\text{H}$ /liter), but this method was abandoned because of lethal effects on the plants and insufficient specific radioactivity (0.5  $\mu\text{Ci}/\text{mg}$ ) in the purified PHA for precise electron microscopic autoradiography. Preliminary results of direct labeling techniques such as  $^3\text{H}$ -acetylation of the purified mitogens has yielded much higher specific radioactivity (up to 7 to 10  $\mu\text{Ci}/\text{mg}$ ) with no loss of biological activity, and this method will be pursued. Successful fluorescent labeling by techniques such as dansylation of the mitogens is also being accomplished.

## 4. Localization of the Mitogenic Molecules in Lymphocytes.

The degree of extra-cellular, cell membrane, and intracellular localization of the labeled mitogens at various times after culture will be determined by autoradiographic techniques (bright field and electron microscopy). In our preliminary work we found largely cytoplasmic localization of the label. The use of purified mitogens with higher specific radioactivity should allow precise localization at the organelle level by autoradiography with electron microscopy. Subcellular fractions (obtained by cell disruption with sonic, freeze-thaw, or homogenization techniques) will be radiologically assayed at different times after culture to determine localization of the label. Preliminary studies indicate localization largely in the mitochondrial fraction.

## 5. Mechanisms of Action of the Mitogenic Molecules.

Formation of mitogen complexes with cellular elements such as RNA and DNA will be studied (1) on lymphocyte smears treated with enzymes (RNase, DNase) to determine integrity of label and (2) by direct chemical separation of RNA and DNA from whole cells and subcellular fractions for radiological assay.

Sequential studies will be made of the site of the mitogenic label in cells as related to changes in the metabolism of RNA, DNA, proteins, etc. RNA and DNA metabolism in lymphocytes will be observed by pulse-labeling with radioactive nucleoside precursors such as tritiated cytidine, thymidine, and/or uridine and measuring the incorporation of radioactive label at various times after mitogenic

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stimulation of the cells.

Separated, purified organelle fractions (particularly mitochondria) will be studied for (1) changes in metabolism of the organelles from treated cultures and (2) direct effects of the mitogen on the separated organelles from untreated cultures with regard to labeling and metabolism.

Structural modifications of nuclear histones such as acetylation appear to follow quickly a triggering mechanism of the mitogen (from the cytoplasm?), resulting in derepression of the DNA. We plan to measure acetylation quantitatively and temporally in mitogen-stimulated lymphocytes. Attempts will be made to correlate histone acetylation and RNA and DNA synthesis as well as cytoplasmic organelle changes with mitogenic action.

The above techniques and the findings on normal lymphocytes will be applied to the study of possible abnormal mechanisms in the lymphocytes in CLL and other disorders with loss of immunological competence.

#### D. Significance

The lymphocyte is emerging as an important immunocyte in the body and appears to be the critical mediator in antigenic recognition (immunologic memory), delayed hypersensitivity, and homograft rejection. Altered function of the lymphocyte probably occurs in diseases associated with immunologic deficiency such as chronic lymphocytic leukemia, autoimmune diseases, etc.

Intracellular changes associated with lymphocyte transformation by mitogens are poorly understood, particularly at the molecular level. The use of a mitogenic molecule isolated in pure form from PHA and radioactively labeled to investigate intracellular mechanisms associated with lymphocyte transformation and proliferation should furnish information of significance in the following respects:

1. The elucidation of such intracellular mechanisms should further our knowledge of basic immunologic reactions at the cellular level.
2. Though the mitogen from PHA causes widespread transformation and proliferation of lymphocytes (generally believed to be a nonspecific effect), the mechanisms may shed light on the intracellular action of more specific antigens.
3. An understanding of the alteration of these mechanisms in lymphocytes in CLL and other disorders associated with immunologic deficiency would be of great importance. Elucidation of metabolic defects associated with these disorders might aid in understanding the etiology and nature of the abnormalities and possibly indicate therapeutic considerations.
4. Studies of mitogen-stimulated histone-DNA interactions may lead to information on the transcription of messenger RNA's. The correlation between RNA synthesis and histone modification in this connection is of great importance to a better understanding of the processes involved in gene activation.
5. PHA as generally used has not been in a pure form but has contained several biologically active components. If we can obtain the mitogen in pure form, we will be able to study the basic molecular configuration of the biologically active moiety of the mitogenic molecules and thus help elucidate the molecular reactions involved. Also, perfection of techniques for isolation and identification of PHA

components will be of value in the study of other protein systems.

6. In the continuing search for a possible virus associated with human leukemia, the enhancing action of PHA mitogens on virus may be an important factor in identifying virus if present.

#### E. Facilities Available

Two well equipped laboratories with office space are available for work with cultures and physical-chemical separation techniques.

The 48-bed research hospital and the outpatient clinic associated with our Medical Department afford a source of blood samples for the normal, aging, and leukemic blood studies.

The following special equipment is available either in our laboratories or nearby:

1. Facilities for cell culture with large capacity incubators.
2. Coulter F electronic counter; 250 channel electronic cell sizing equipment.
3. Microscopes and analytical balances.
4. Refrigerated laboratory for low temperature work.
5. Beckman DB-GT spectrophotometer.
6. LKB-Uvicord II, automatic, continuous flow, UV recording spectrophotometer.
7. Cary 15 automatic recording spectrophotometer.
8. Fraction collectors on mobile mount.
9. Complete paper and gel electrophoretic equipment, analytical and preparative.
10. Thin-layer chromatographic equipment.
11. Gas chromatographic equipment.
12. Mechanically refrigerated lyophilizer.
13. Automatic Servall refrigerated superspeed centrifuge.
14. Spinco preparative ultracentrifuge, Model L.
15. Analytical ultracentrifuge, Spinco Model E.
16. Counters for radioactive measurements (liquid scintillation, etc.)
17. Electron microscope.
18. Two Beckman/Spinco amino acid analyzers.
19. Circular dichroism spectropolarimeter.
20. Complete photographic services.

#### F. Appendix

Material appended.

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